IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re the Application of:	) Group Art Unit: 1646
	)
COX et al.	) Examiner: Xie, Xiaozhen
	)
Serial No.: 10/031,154	) **DECLARATION OF**
	) **GEORGE COX AND DANIEL DOHERTY**
Filed: January 14, 2002	) **UNDER 37 CFR 1.131**
	)
Atty. File No.: 4152-3-PUS	)
	)
For: "IMMUNOGLOBULIN FUSION	)
PROTEINS"	)

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

　　　　We, George Cox and Daniel Doherty, each declare as follows:

　　　　1.　　　I am a co-inventor of the above-referenced patent application and am familiar with the application.

　　　　2.　　　This Declaration under 37 CFR §1.131 is being submitted in conjunction with a Response to an Office Action mailed on June 27, 2006, the Response being filed herewith.

　　　　3.　　　This Declaration provides factual evidence that the invention as claimed in *at least* Claims 90, 91 (in part), 92 (in part), 94 (in part), 96, 104, 130, 133, 134 and 135 of the above-identified patent application was *actually reduced to practice* prior to the publication date of January 21, 1999 of PCT Publication No. WO 99/02709 by Sytkowski. All acts relied upon to establish the dates of actual reduction to practice were carried out in the United States.

Evidence of Actual Reduction To Practice

　　　　The invention presently claimed in at least Claims 90, 91 (in part), 92 (in part), 96, 104, 130, 133, 134, 135, 136 and 138 of the above-identified patent application was actually reduced

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to practice by a date prior to January 21, 1999, which is the publication date of PCT Publication No. WO 99/02709.

As evidence of actual reduction to practice by a date prior to January 21, 1999, enclosed as Exhibit A are notebook pages from an experiment that was completed by us prior to January 21, 1999. This experiment demonstrates that three different EPO-Ig fusion proteins that had been constructed and produced by us had significant biological activity as demonstrated using a UT7/epo cell proliferation bioassay. Each of the fusion proteins comprised erythropoietin joined at its carboxy-terminus by a peptide linker of between 2 and 7 glycine and/or serine amino acid residues to the amino terminus of an immunoglobulin domain that does not contain a variable region.

The three fusion proteins are denoted pBBT 179, pBBT 180 and pBBT 181. Construct pBBT 179 is EPO-IgG$_1$/CH$_1$ (EPO-IgG$_1$); construct pBBT 180 is EPO-IgG$_1$/hinge (IgG$_1$-Fc); and construct pBBT 181 is EPO-IgG$_4$/hinge (IgG$_4$-Fc). In these fusions, the peptide linker was 7 amino acids in length (Claims 90, 92, 133, 134, 135, 136 and 138) and consisted of glycine and serine residues (Claims 90, 92, 133, 134, 135, 136 and 138). The Ig portion of the fusion protein was either IgG1-Fc, IgG4-Fc or IgG1-CH (Claims 91 and 130), demonstrating that we had produced fusion proteins using two different Ig isotypes and both Fc and CH fusions. Prior to the experiment described in Exhibit A below, the three fusion protein nucleotide constructs were produced using recombinant techniques, and the fusion proteins were expressed by transfecting a host cell with an expression vector comprising the recombinant constructs, culturing the host cell under conditions effective to express the fusion protein, and harvesting the fusion protein expressed by the host cell (Claim 104). The expression of the fusion proteins was demonstrated and levels of expression quantitated by Western blots using anti-EPO antibodies in order to determine the concentrations of the proteins prior to putting them into the bioassay described in Exhibit A below (data not shown in this Declaration).

The EC$_{50}$'s (as defined in the present application, the concentration of protein required for half-maximal stimulation) of each of the three EPO-Ig fusion proteins was measured in the UT7/epo cell proliferation bioassay, shown in the notebook pages of Exhibit A. The first page of the Exhibit shows the three fusion proteins tested as discussed above (BBT 179, BBT 180, BBT

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181), with plasmid pCDNA3.1 serving as a negative control and a wild type human EPO protein purchased from R & D Systems, Inc. serving as a positive control. Plasmids BBT179, BBT180 BBT181 and pcDNA3.1 were used to transfect COS cells and the conditioned media containing the fusion proteins was harvested several days later. Serial 10-fold dilutions of the conditioned media were prepared and assayed in the UT7/epo cell proliferation assay. The serial dilutions were called tubes 1-6. Estimated concentrations of the EPO-IgG fusion proteins in the assay were: tube 1, 0.0005 ng/mL; tube 2, 0.005 ng/mL; tube 3, 0.05 ng/mL; tube 4, 0.5 ng/mL; tube 5, 5 ng/mL; and tube 6, 50 ng/mL. Six serial dilutions of the EPO control protein were prepared as well. Concentrations of the EPO standard in the assay were: tube 1, 0.0004 ng/mL; tube 2, 0.004 ng/mL; tube 3, 0.04 ng/mL; tube 4, 0.4 ng/mL; tube 5, 4 ng/mL and tube 6, 40 ng/mL. The second page of Exhibit A shows the experimental set-up for each of the three test plates (called plates A, B and C). Each of the serial dilutions was assayed in triplicate. Additional control wells contained no cells (called "no cells") or just media but no Epo or Epo-Ig (called "0"). The UT7/epo cell line shows a strong proliferative response to rEPO, as evidenced by a dose-dependent increase in absorbance values, which is proportional to cell number. In the absence of rEPO, the majority of UT7/epo cells die, giving absorbance values less than 0.1. The results shown on the third-sixth pages of the Exhibit provide the raw data and graphs of the activity of the three EPO-Ig fusion proteins as compared to rEPO and a negative control. The graphs plot absorbance of the wells on the Y-axis versus the dilution tube on the X-axis (the percent of the COS cell supernatant (% sup) in the dilution tubes for the fusion proteins or the number of EPO units/mL (1 unit 8 ng/mL) in the dilution tubes also is plotted on the X-axis under the appropriate dilution tube). The dilution tube closest to the EC_{50} for each fusion protein was tube 4, which contained an estimated 0.5 ng/mL of the fusion protein. The serial dilution tube closest to the EC_{50} for the EPO control protein also was tube 4, which contained an estimated 0.4 ng/mL of EPO. Thus, the EC_{50}s of the fusion proteins were within the scope of less than 4 ng/ml, and comparable to (within at least 4 fold) activity of wild type EPO on a molar basis, which also represents an EC_{50} of less than 1000 ng/ml and less than 10 ng/ml (Claims 96, 134, 135, and 136).

This factual evidence is believed to be sufficient to establish an actual reduction to

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practice of the invention as claimed in at least Claims 90, 91 (in part), 92 (in part), 96, 104, 130, 133, 134, 135, 136 and 138 at a date prior to January 21, 1999.

    4.   This Declaration also provides factual evidence of the conception of the invention as claimed in Claims 67, 68, 77, 78, 80-87, 89-94, 96, 102, 104, 105, and 125-138, at a date prior to the January 21, 1999 publication date of PCT Publication No. WO 99/02709 by Sytkowski, followed by diligence in reduction to practice from a date prior to the earliest publication date of PCT Publication No. WO 99/02709 by Sytkowski (January 21, 1999) to the date of constructive or actual reduction to practice of Claims 67, 68, 77, 78, 80-87, 89, 91 (in part), 92 (in part), 93, 94, 102, 105, 125-129, 131, 132 and 137. As demonstrated in paragraph 3 above, at least Claims 90, 91 (in part), 92 (in part), 96, 104, 130, 133, 134, 135, 136 and 138 were actually reduced to practice at a date prior to January 21, 1999. All acts relied upon to establish the dates of conception, diligence and reduction to practice were carried out in the United States.

Evidence of Conception prior to January 21, 1999

    (1) As evidence of conception of the invention as claimed in Claims 67, 68, 77, 78, 80-87, 89-94, 96, 102, 104, 105, and 125-138, at a date prior to the January 21, 1999 publication date of PCT Publication No. WO 99/02709, enclosed as Exhibit B are relevant portions of a grant application that was prepared by us and submitted at a date prior to January 21, 1999.

    This grant application proposes the construction and production of Epo-Ig fusion proteins (all claims), including Epo/IgG-Fc and Epo/IgG-CH fusion proteins (Claims 68, 91, and 130), with biological activities (EC_{50}'s) that are comparable to wild-type EPO on a molar basis, which is encompassed by all of the EC_{50} values claimed (Claims 96, 126, 128, 129, 134, 135, 136 and 137). The grant sections describe joining the C-terminus of EPO to the N-terminus of human IgG-Fc or IgG-CH domains using a restriction site to join the protein domains (Claims 67, 90, 125, 126, 137 and dependents therefrom). The grant describes the production of these proteins for use as therapeutic proteins that prolong the circulating half-life of the protein *in vivo*, such as for treating anemia, with respect to EPO (Claims 77, 87 and 89).

    More specifically, the grant sections provided show the amino acid sequence and structural organization of human EPO (Claim 127) and provide details for the production of the constructs using unique restriction sites to join the EPO and IgG-Fc or IgG-CH sequences

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(Claims 67, 68, 90, 91, 125, 126, 137 and dependents therefrom). On page 11, lines 14-22 of the application, we state that the junction sequences (peptide linkers) will be the only non-natural sequences in the fusion protein, thereby teaching the use of a linker to connect the proteins (Claims 90, 92-94, 131-136 and 138), and we also state that the non-natural sequences (peptide linkers) can be removed by *in vitro* mutagenesis to create fusions containing only human sequences, thereby teaching an EPO-Ig fusion protein without an intervening peptide linker (Claims 67, 125, 126, 127, and 137). We describe how we will amplify the genes encoding EPO and the Ig portions of the fusion protein using, *e.g.*, PCR, and clone them into mammalian cell expression vectors (Claims 80-85 and Claim 104). We discuss how to express proteins in mammalian COS or CHO cells (Claim 82-83). We state that we expect the proteins to be secreted as homodimers, and we state that we will purify the proteins by affinity chromatography using protein A columns (Claims 78, 85, 86, 102, 104, 105). We state that fusion proteins can be further purified by ion-exchange, size-exclusion or hydrophobic interaction column chromatography (Claims 85, 86). We state that bioactivities of the proteins can be measured using the UT7/epo cell line and again, propose EPO-Ig fusions with biological activities comparable to wild-type EPO (Claims 96, 126, 128, 129, 134, 135, 136 and 138). We describe how *in vivo* activity of the proteins can be measured in rodent models for anemia (low hematocrit) (Claims 87 and 89).

(2) As further evidence of conception of the invention as claimed in at least Claims 90, 91, 92, 96, 104, 130, 133, 134, 135, 136 and 138 at a date prior to the January 21, 1999 publication date of PCT Publication No. WO 99/02709, Exhibit A, discussed in detail in paragraph 3 above, is referenced. This Exhibit also demonstrates conception, in addition to the actual reduction to practice, of three different EPO-Ig fusion proteins that were also encompassed in the conception of the invention as described in Exhibit B discussed above, and specifically, EPO-IgG fusion proteins that contain a peptide linker within the scope of Claim 90 and related dependent claims, which is also the starting template for the EPO-IgG fusion proteins that do not contain an intervening linker (Claim 67, 125, 126 and 137), noting that Exhibit B proposes to remove the linkers from the linkered fusions by site-directed mutagenesis to create the direct fusions.

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Evidence of Conception and Diligence beginning prior to January 21, 1999

(1) Exhibit A also represents an experiment completed prior to January 21, 1999 (*i.e.*, on January 12-15, 1999) which provides evidence that we were diligently performing experiments to reduce the invention as claimed in Claims 67, 68, 77, 78, 80-87, 89-94, 96, 102, 104, 105, and 125-138 to practice. In particular, this experiment establishes that we were diligently performing experiments to reduce to practice the construction, production, and testing of biologically active EPO-Ig fusions, beginning with EPO-Ig fusions having the recited small peptide linkers consisting of serine and glycine amino acids, which were also the first step in producing the direct fusions (EPO-Ig without an intervening peptide linker).

(2) Exhibit C also represents an experiment completed prior to January 21, 1999 (*i.e.*, October 1-9, 1998) that provides additional evidence that we were diligently performing experiments to reduce the invention as claimed in Claims 67, 68, 77, 78, 80-87, 89-94, 96, 102, 104, 105, and 125-138 to practice. Specifically, these notebook pages describe the planning of different EPO-Ig fusion constructs and then the initial production of constructs encoding immunoglobulin and EPO proteins that were used to produce various EPO-Ig fusion proteins. These constructs were used to produce the linkered fusion proteins, which were in turn used as starting templates from which the fusions without an intervening linker were produced. Specifically, these notebook pages show diagrams for fusion constructs of EPO and IgG$_1$ and IgG$_4$ (Fc or hinge and CH domains), as well as marked up sequences for EPO and IgG$_1$ and IgG$_4$, and oligonucleotide design, as well as experiments showing successful cloning and expression of the individual components that were used to create EPO-Ig fusion proteins as claimed.

Evidence of Continued Diligence and Constructive and/or Actual Reduction to Practice

Between January 15, 1999 and July 13, 1999, we continued to design constructs and perform experiments to produce and test additional EPO-Ig fusion proteins as claimed in Claims 67, 68, 77, 78, 80-87, 89-94, 96, 102, 104, 105, and 125-138, and we worked with patent counsel to constructively reduce the invention to practice by the preparation and filing of U.S. Provisional Application No. 60/143,458, filed July 13, 1999, which is the priority document for the present application. The following Exhibits describe activities and representative experiments that pertained to the constructive and actual reduction to practice of the invention

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during this time period.

(1) Exhibit D contains notebook pages dated from February 9-17, 1999, showing the results of an experiment that began on January 21, 1999 (see reference to 1/21/99 transfection), showing the larger scale transfection of host cells with the EPO-Ig fusion constructs described in the Exhibits above and the purification of the fusion proteins (purification of EPO-IgG$_4$/hinge (Fc) or pBBT 181, EPO-IgG$_1$/CH or pBBT 179, and EPO-IgG$_1$/hinge (Fc) or pBBT 180 is shown).

(2) Exhibit E contains a notebook page date February 26, 1999, showing the design and beginning of the construction of a recombinant construct encoding a different EPO-Ig fusion protein, which was an EPO joined at its carboxy-terminus to IgG$_4$-CH (an immunoglobulin domain that does not contain a variable region) using a linker comprised of serine and glycine residues. Note the diagram illustrating the construct plan.

(3) Exhibit F contains notebook pages showing an experiment beginning on March 23, 1999, and ending on April 28, 1999, describing the COS cell transfection, expression and purification of the new EPO-Ig fusion protein (EPO-IgG$_4$/CH (pBBT 185)) described in Exhibit E above.

(4) Exhibit G provides relevant portions of a grant application completed and filed on April 14, 1999, which includes particular experimental details regarding the construction and production of EPO-Ig fusion proteins having 2 and 4 amino acid linkers, and EPO-Ig fusions having no intervening linker. This grant also includes much of the material described in the grant sections of Exhibit B and presents the data shown in Exhibit A and related experiments and specifically, describes the detailed construction, production and testing of EPO-IgG-Fc and EPO-Ig-CH fusions with a peptide linker. This document also describes specific methods for separating monomers from dimers.

(5) Exhibit H contains notebook pages showing the results on June 8-9, 1999, of an experiment that began April 30, 1999, showing the purification of a large scale EPO-IgG$_1$-Fc fusion protein (pBBT 180) transfection experiment.

(6) Exhibit I contains a notebook page from an experiment performed on July 6-9, 1999, showing the bioactivity for the EPO-IgG$_4$-CH fusion protein (pBBT 185) and for the scaled up

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expression of the EPO-IgG$_1$-Fc fusion protein (pBBT 180). The EC$_{50}$s for the fusion proteins are shown as compared to wild-type recombinant EPO. The EC$_{50}$ for the EPO control protein was 0.48 and 0.48 ng/mL in two assays; the EC$_{50}$s for BBT185 were 2 and 2.1 ng/mL in two assays and the EC$_{50}$s for BBT180 were 1.3 and 1.3 ng/mL in two assays.

(7) Exhibit J is U.S. Provisional Application Serial No. 60/143,458, filed July 13, 1999, which constructively reduces to practice the invention as claimed in Claims 67, 68, 77, 78, 80-87, 89-94, 96, 102, 104, 105, and 125-138.

5.   I hereby declare that all statements made herein of my own are true and that all statements made on information and belief are believed to be true; and further that the statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the subject application or any patent issuing therefrom.

August 20, 2007
Date
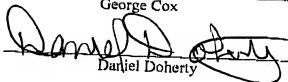
George Cox

August 20, 2007
Date

Daniel Doherty

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EXHIBIT A

from Page No._____

Elim-X Column to Sterilize   do 10x dilution the time
  BGT 179 - EPO IgG1 CH1   to get ↑ S:B trans report
       180 - EPO Ig61 Fc   w/ 3x dilutions
       181 - EPO Ig G4 Fc
  pCDNA3.1 control

R+D EPO as control

WT   (Assay)   (Assay)
                                     (15591)   (ng/ml)
  54 -> 500 ml = 10u/ml = 2 = 5-u/ml   5   6   .0
  50 -> 500   0.5   4   5   .4
  50 -> 50   0.05   3   4   .4
  50 -> 50   0.005   2   3   .04
  50 -> 500   0.0005   2
  50 -> 500   1

COS cell s-as   if 1 ng/ml
                                         (Fine)
  30 ml -> 300 ml   5   6   50 ng/ml
  50 ml -> 500   5   5   5-
  50   -> 500   4   4   0.5
  50   -> 500   3   3   0.05
  50   -> 500   2   2   0.005
  50   -> 500 50 ->500 1   1   0.0005
  50   -> 500
  50   -> 500

CT cell = 117 := 1 field => 1.17 x 10⁶ /ml
         d 1 ml EPO
         with 3+
         r5p 11.7 ml media

Witnessed & Understood by me,   Date   Invented by Joe L   Date
                                 7/26/99   Recorded by   1/12/99

From Page No.___

A   B   C

     179   181   3.1
                       #
     180   #T
                       UT

                                    T 37   10 p~

clright dow fpo - set q ×st ul out q it j s al chrt

     50 ul cell
     50 ul media   [Ende~ w/ P R
                     [10% FBS   Fresh
                     [Glu; G /

     No cell _o   ,   2   3   4   5   6

                     1/15/59   MTS ~ 11:30

                     Real ~ 2:30 3 hr

                          Go to page 95 And date

Witnessed & understood by me,	Date 1/26/99	Invented by	Date
		Recorded by	1/12/99

From β887

Elo-Ip Fusions

TEST NO.	:	W/L MODE	: DUAL	DATE	: 01/15/99
TEST NAME	: MTS19498	TEST FILTER	: 490 nm	TIME	: 14:29:42
PLATE	: JC11599A17918	REF. FILTER	: 650 nm	OPERATOR	:

Calculation mode : Endpoint *plate A* 179 / 180

DATA MATRIX : OD

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.197	0.199	0.193	0.196	0.192	0.196	0.197	0.195	0.196	0.195	0.197	0.195
B	0.196	0.241	0.278	0.287	0.297	0.302	0.428	0.996	1.108	0.191	0.191	0.194
C	0.194	0.229	0.270	0.289	0.295	0.308	0.422	1.002	1.121	0.192	0.193	0.193
D	0.194	0.238	0.283	0.292	0.298	0.302	0.415	0.997	1.133	0.192	0.190	0.190
E	0.191	0.253	0.272	0.276	0.282	0.306	0.540	1.106	1.128	0.192	0.189	0.193
F	0.190	0.247	0.279	0.279	0.279	0.309	0.542	1.099	1.116	0.189	0.189	0.189
G	0.193	0.244	0.262	0.279	0.284	0.305	0.548	1.099	1.121	0.189	0.188	0.191
H	0.187	0.192	0.183	0.187	0.191	0.193	0.190	0.194	0.192	0.189	0.188	0.187

(B row annotations: N cells 0 1 2 3 4 5 6 ; 179 ; 180)

175   180

10 cells $\bar{x} = 0.236$

0	$\bar{x} = 0.277 - 0.236 = 0.041$	+/- 0.007
1	$\bar{x} = 0.289 = 0.053$	+/- 0.003
2	$\bar{x} = 0.297 = 0.061$	+/- 0.001
3	$\bar{x} = 0.304 = 0.068$	+/- 0.003
4	$\bar{x} = 0.422 = 0.186$	+/- 0.007
5	$\bar{x} = 0.998 = 0.762$	+/- 0.003
6	$\bar{x} = 1.121 = 0.884$	+/- 0.013

No cells $\bar{x} = 0.248$

0	$\bar{x} = 0.271 - 0.248 = 0.023$	+/- 0.009
1	$\bar{x} = 0.278 = 0.030$	+/- 0.002
2	$\bar{x} = 0.282 = 0.034$	+/- 0.003
3	$\bar{x} = 0.307 = 0.059$	+/- 0.002
4	$\bar{x} = 0.543 = 0.295$	+/- 0.004
5	$\bar{x} = 1.101 = 0.853$	+/- 0.004
6	$\bar{x} = 1.122 = 0.874$	+/- 0.006

Witnessed & Understood by me, Date 1/26/99
Invented by _____
Recorded by _____   Date 1/15/99

Project No._____
Book No._____ TITLE _Epo Ief Fusion_   Pg 87

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From Page No._____

TEST NO.   :   W/L MODE   : DUAL   DATE   : 01/15/99
TEST NAME   : MTS19498   TEST FILTER : 490 nm   TIME   : 14:32:27
PLATE   : JC11599B181wt REF. FILTER : 650 nm   OPERATOR :

Calculation mode   : Endpoint   Plate B   181
                                                        ESA — wt epo

DATA MATRIX : OD

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.182	0.193	0.188	0.195	0.187	0.192	0.188	0.190	0.185	0.186	0.196	0.198
B	0.189	0.239	0.285	0.288	0.305	0.340	0.748	1.208	1.236	0.194	0.194	0.194
C	0.185	0.241	0.279	0.298	0.304	0.343	0.737	1.212	1.264	0.192	0.193	0.193
D	0.183	0.248	0.280	0.297	0.303	0.337	0.724	1.280	1.234	0.192	0.196	0.142
E	0.182	0.244	0.284	0.281	0.300	0.341	0.685	1.186	1.242	0.195	0.195	0.193
F	0.177	0.244	0.271	0.286	0.293	0.338	0.682	1.180	1.306	0.193	0.182	0.194
G	0.179	0.246	0.265	0.287	0.298	0.340	0.673	1.187	1.253	0.191	0.191	0.192
H	0.176	0.194	0.181	0.187	0.189	0.191	0.192	0.194	0.190	0.190	0.192	0.190

Cells: 0, 1, 2, 3, 4, 5, 6 (handwritten above row B columns)

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wt epo

No cells $\bar{x} = 0.243$

0	$\bar{x} = 0.281 - 0.243 = 0.038 +/- 0.003$	
1	$\bar{x} = 0.294$ $= 0.051 +/- 0.006$	
2	$\bar{x} = 0.304$ $= 0.061 +/- 0.001$	
3	$\bar{x} = 0.340$ $= 0.097 +/- 0.003$	
4	$\bar{x} = 0.736$ $= 0.493 +/- 0.012$	
5	$\bar{x} = 1.210$ $= 0.967 +/- 0.003$	
6	$\bar{x} = 1.245$ $= 1.002 +/- 0.017$	

No epo $\bar{x} = 0.245$

0	$\bar{x} = 0.273 - 0.245 = 0.028 +/- 0$	$= 0.042 +/- 0$
1	$\bar{x} = 0.285$ $= 0.0 +/- 0$	
2	$\bar{x} = 0.297$ $= 0.052 +/- 0.0$	
3	$\bar{x} = 0.400$ $= 0.095 +/- 0.$	
4	$\bar{x} = 0.680$ $= 0.435 +/- 0.0$	
5	$\bar{x} = 1.184$ $= 0.939 +/- 0.0$	
6	$\bar{x} = 1.267$ $= 1.022 +/- 0.0$	

To Page No._____

Witnessed & Understood by me,   Date   Invented by [signature]   Date
[signature]   3/26/99   Recorded by [signature]   1/15/99

TEST NO.   :   W/L MODE   : DUAL   DATE   : 01/15/99
TEST NAME   : MTS19498   TEST FILTER : 490 nm   TIME   : 14:35:07
PLATE   : JC 11599C3   REF. FILTER : 650 nm   OPERATOR   :

Calculation mode   : Endpoint.   glut C   βCDNA 3.1

DATA MATRIX : OD

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.190	0.197	0.193	0.194	0.195	0.197	0.196	0.193	0.198	0.193	0.194	0.195
B	0.197	0.247	0.294	0.303	0.309	0.312	0.313	0.313	0.304	0.197	0.193	0.193
C	0.197	0.253	0.301	0.308	0.314	0.308	0.323	0.309	0.308	0.193	0.193	0.194
D	0.187	0.258	0.299	0.307	0.311	0.314	0.317	0.311	0.304	0.195	0.197	0.195
E	0.194	0.196	0.189	0.191	0.192	0.191	0.193	0.192	0.192	0.195	0.192	0.193
F	0.195	0.196	0.184	0.190	0.191	0.190	0.195	0.194	0.194	0.193	0.192	0.188
G	0.192	0.192	0.179	0.186	0.190	0.189	0.191	0.196	0.191	0.193	0.193	0.185
H	0.170	0.193	0.185	0.184	0.192	0.194	0.195	0.191	0.194	0.193	0.184	0.189

In row B above columns 2–9 handwritten: "cells 0 1 2 3 4 5 6"

βCDNA 3.1

No cells $\bar{x}=$ 0.253

0 $\bar{x}=$ 0.298 − 0.253 = 0.045 +/− 0.004
1 $\bar{x}=$ 0.306   = 0.053 +/− 0.003
2 $\bar{x}=$ 0.311   = 0.0589 +/− 0.003
3 $\bar{x}=$ 0.311   = 0.058 +/− 0.003
4 $\bar{x}=$ 0.318   = 0.065 +/− 0.005
5 $\bar{x}=$ 0.311   = 0.058 +/− 0.002
6 $\bar{x}=$ 0.305   = 0.052 +/− 0.002

nessed & Understood by me,   Date 7/26/99   Invented by _____   Date 1/14/99
Recorded by _____

Result:

EPO-IgG's are very active. If Denis estimate of IgG lvl
is correct, then EC50's ~ same as wt EPO

Read & Understood by me,	Date 2/26/99	Invented by	Date 1/15/99
		Recorded by	

EXHIBIT B

*A. Specific aims:
*   There is considerable interest on the part of patients and healthcare providers in the development of
*low cost, long-acting, "user-friendly" protein therapeutics. Most protein pharmaceuticals have short
*circulating half-lives in the body and must be injected daily or every other day for maximum effectiveness.
*For example, growth hormone (GH) and granulocyte colony-stimulating factor (G-CSF) require daily
*injections and erythropoietin (EPO) requires every other day injections for maximum effectiveness. These
*recombinant proteins have proven extremely effective at treating short stature and cachexia (GH),
*neutropenia (G-CSF) and anemia (EPO). For each of these proteins it is known that increasing the
*circulating half-life of the protein improves the protein's in vivo performance. We propose to create
*longer-acting forms of GH, G-CSF and EPO through covalent fusion of these proteins to the heavy chain
*domain of human IgG1. Human IgG1 has a long serum half-life, on the order of 21 days. Fusion of
*several other proteins, principally extracellular domains of cell surface receptors, to the IgG1 heavy chain
*domain has resulted in increased serum half-lives for these proteins. Despite this success, IgG fusion
*protein technology has been applied in only a few instances to cytokines and growth factors. Fully active
*IL-2- and IL-10-IgG fusion proteins have been constructed, but data are not available for other cytokines
*and growth factors.
*   During Phase I, we will create recombinant fusion proteins comprising GH, G-CSF or EPO fused to
*the Fc (Hinge- CH2-CH3) or complete heavy chain (CH1-Hinge- CH2- CH3) domains of human IgG1.
*The fusion proteins will be expressed as secreted proteins from transiently transfected mammalian cells or
*Baculovirus-infected insect cells. The proteins will be purified by Protein A or G affinity chromatography
*and their bioactivities compared to the corresponding non-fusion proteins in in vitro cell proliferation
*assays. Fusion proteins that retain full in vitro bioactivity will be candidates for further development.
*   During the Phase II portion of the grant, we will develop manufacturing processes to produce sufficient
*quantities of the fusion proteins for pharmacokinetic analyses and for testing in animal disease models.
*Our goal is to create fusion proteins that are equal or superior to the natural proteins in stimulating
*biological activities in vivo, but which require less frequent dosing, on the order of once every two to four
*weeks, rather than daily or every other day. Previous studies suggest it should be possible to achieve this
*goal by fusion of the proteins to the heavy chain domain of human IgG1.
*   The primary goal of the Phase I portion of the grant is to identify one or more IgG fusion proteins of
*GH, G-CSF or EPO that possesses wild type in vitro biological activity. The specific tasks involved are:

*1. Clone cDNAs encoding GH, G-CSF and EPO and fuse DNA encoding these proteins to DNA encoding
*the Fc or complete heavy chain (C_H) region of human IgG1.

*2. Clone DNA encoding the fusion proteins into a mammalian cell or insect cell expression vector.

*3. Transiently transfect mammalian cells with DNA encoding the fusion proteins and purify the secreted
*fusion proteins to homogeneity using Protein A or G affinity chromatography, followed by other
*chromatographic procedures, if needed. Alternatively, express the fusion proteins in insect cells and
*purify the secreted proteins as described above.

*4. Characterize the fusion proteins by polyacrylamide gel electrophoresis under reducing and non-reducing
*conditions to determine whether the fusion proteins are dimeric. Determine the effective sizes of the
*fusion proteins using size-exclusion chromatography.

*5. Measure bioactivities (EC_{50}s) of the fusion proteins using appropriate GH-, G-CSF-, and EPO-
*responsive mammalian cell lines in culture. Bioactivities of the fusion proteins will be compared to the
*bioactivities of the non-fused proteins.

*   Fusion proteins that retain full biological activity will be candidates for further development during the
*Phase II portion of the grant. These studies will entail:

*6. Develop stable cell lines expressing the fusion proteins and purify sufficient quantities of the fusion
*proteins for animal studies.

*7 Perform pharmacokinetic experiments to demonstrate increased circulating half-lives of the fusion
*proteins.

*8 Perform experiments in animal disease models to compare the relative efficacies of the fusion proteins to
*the corresponding non-fusion proteins.

*B. Significance
*   There is considerable interest on the part of patients and healthcare providers in the development of
*low cost, long-acting, "user-friendly" protein therapeutics. Proteins are expensive to manufacture and
*unlike conventional small molecule drugs, are not readily absorbed by the body. Therefore, proteins must
*be administered by injection. Most proteins are cleared rapidly from the body, necessitating frequent,
*often daily, injections for optimum effectiveness. This is the case for GH and G-CSF. Some proteins such
*as erythropoietin (EPO) are effective when administered less often (three times per week for EPO) but this
*is due to the fact that the proteins are glycosylated, which requires that they be produced using expensive
*mammalian cell expression systems. Patients dislike injections, which leads to reduced compliance and
*reduced drug efficacy. The length of time an injected protein remains in the body is finite and is
*determined by the protein's size and whether or not the protein contains covalent modifications such as
*glycosylation. Circulating concentrations of injected proteins change constantly, often by several orders
*of magnitude, over a 24 hour period. Rapidly changing concentrations of protein agonists can have
*dramatic downstream consequences, at times understimulating and at other times overstimulating target
*cells. Similar problems plague protein antagonists. These fluctuations can lead to decreased efficacy and
*increased frequency of adverse side-effects for protein therapeutics. The rapid clearance of recombinant
*proteins from the body significantly increases the amount of protein required per patient and dramatically
*increase the cost of treatment. The cost of human protein pharmaceuticals is expected to increase
*dramatically in the years ahead as new and existing drugs are approved for more disease indications.
*Within 10 years the sequence of the human genome will be known, unleashing a flood of potential new
*protein therapeutics. Current word-wide sales of protein therapeutics are in excess of $10 billion annually
*and are growing at a greater than 10% annual rate. Thus, there is a strong need to develop protein
*delivery technologies that lower the costs of protein therapeutics to patients and healthcare providers. One
*solution to this problem is the development of methods to prolong the circulating half-lives of protein
*therapeutics in the body so that the proteins do not have to be injected frequently. This solution also
*satisfies the needs and desires of patients for protein therapeutics that are " user-friendly", i.e., protein
*therapeutics that do not require frequent injections.

*Extending protein half-life by fusion to human IgGs
*   A second method that has been used to prolong the circulating half-lives of proteins is to use
*recombinant DNA technology to covalently fuse the protein of interest to a second protein that naturally
*has a long circulating half-life. One protein that has a long circulating half-life and which has been used
*to create numerous fusion proteins is human IgG1. IgG1 is the most common immunoglobulin in serum
*(70% of total IgG), has a serum half-life of 21 days (Capon et al., 1989).
*   Human IgGs have a multidomain structure, comprising two light chains disulfide-bonded to two heavy
*chains. Each light chain and each heavy chain contains a variable region joined to a constant region. The
*variable regions are located at the N-terminal ends of the light and heavy chains. The heavy chain
*constant region is further divided into CH1, Hinge, CH2 and CH3 domains. The CH1, CH2 and CH3
*domains are discreet domains that fold into a characteristic structure. The Hinge region is a region of

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*considerable flexibility. The various heavy chain domains are encoded by different exons in the IgG genes
*(Ellison et al., 1982).
*   Proteins have been fused to the heavy chain constant region of IgGs at the junction of the variable and
*constant regions (thus containing the CH1-Hinge-CH2-CH3 domains - referred to as the C_H domain) and
*at the junction of the CH1 and Hinge domains (thus containing the Hinge-CH2-CH3 domains - referred to
*as the Fc domain). The IgG heavy chain fusions create larger proteins that are expected to have longer
*circulating half-lives. IgG1 heavy chains normally form disulfide-linked dimers through cystine bonds
*located in the Hinge region. Since the Hinge region will be present, and unaltered in both of the proposed
*fusion proteins, we expect the fusion proteins will be dimeric. For some proteins such as the extracellular
*domains of tumor necrosis factor receptors, dimer formation has provided unexpected benefits because the
*dimeric proteins were found to possess increased affinities for their ligands (Mohler et al., 1993). GH, G-
*CSF and EPO are believed to act as monomers and there is no evidence to suggest that dimerization will
*improve activity. In fact, it is possible that dimerization with decrease bioactivity of the proteins due to
*interference with the proteins binding their cell surface receptors. Whether this is the case is one of the
*questions we expect to answer during Phase I. We believe the answer to this question will be the crucial
*part of the grant that will determine ultimate success of the project for we believe there is sufficient
*evidence with other proteins to suggest that it is highly likely the IgG fusion proteins will have longer
*circulating half-lives than the non-fused proteins.
*   The fusion proteins under development will be non-natural proteins and potentially immunogeneic in
*humans. We hope to minimize this possibility by using human IgG1 as the fusion partner rather than an
*IgG domain from another species. Thus the entire protein will be of human origin. The junction
*sequences joining the two proteins will be the only "non-natural" sequence in the fusion proteins. Any
*non-natural sequences can be removed at a later time, if warranted, by in vitro mutagenesis, as was done
*with a CD4-IgG fusion protein (Capon et al., 1989). The one IgG fusion that has been studied in detail, a
*fusion of a TNF receptor to the Fc region of human IgG1, has proven to be non-immunogenic in humans
*(Moreland et al., 1996; 1997). Thus there is evidence to suggest that IgG fusion proteins will be non-
*immunogenic.

Figure 3. Amino acid sequence and structural organization of human EPO. N-linked and O-linked glycosylation sites are underlined.

```
-27 signal sequence        -1
MGVHECPAWLWLLLSLLSLPLGLPVLG

1         /9 A-HELIX   22/                    A-B loop
APPRLICD SRVLERYLLEAKEA ENITTGCAEHCSLNENITVPDTKVNFYAWKRMEVGQ

/59  B-HELIX      76/  B-C loop      /90  C-HELIX   107/ C-D loop
QAVEVWQGLALLSEAVLR GQALLVNSSQPWE PLQLHVDKAVSGLRSLTT LLRALGAQ

      C-D loop      /132   D-HELIX    152/                166
KEAISPPDAASAAPLR TITADTFRKLFRVYSNFLRGK LKLYTGEACRTGDR
```

EPO Background

*   EPO is the hormone primarily responsible for stimulating erythropoiesis or red blood cell formation.
*EPO acts on immature red blood cell precursors to stimulate their further proliferation and differentiation
*into mature red blood cells. Recombinant human EPO is used to restore red blood cell production in
*patients with anemia resulting from renal failure, chemotherapy and drug complications. EPO recently
*received FDA approval for stimulating red blood cell formation in patients undergoing certain types of
*elective surgeries. U.S. sales of EPO exceeded $1 billion and world-wide sales exceeded $2 billion in
*1996.
*   Human EPO is a 35-39 kDa glycoprotein secreted by the adult kidney. The mature human protein
*contains 166 amino acids and is heavily glycosylated. The sequence of human EPO is shown in Figure 3.
*The primary sequence of EPO is highly conserved among species (greater than 80% identity). Sugar
*groups account for greater than 40% of the protein's mass. Human EPO contains three N-linked
*glycosylation sites and one O-linked glycosylation site (underlined in Figure 3). The N-linked
*glycosylation sites are conserved in different species whereas the O-linked glycosylation site is not.
*Proper glycosylation of the N-linked glycosylation sites in EPO extends the protein's circulating half-life.
*in the body and improves the protein's performance in animal disease models (Fukada et al., 1989; Spivak
*and Hogans, 1989; Delorme et al., 1992).
*   The extensive glycosylation of EPO has prevented the protein's crystallization, so the X-ray structure
*of the protein is not known. The amino acid sequence of EPO is consistent with the protein being a
*member of the GH supergene family (Bazan, 1991; Mott and Campbell, 1995) and mutational studies
*support this view of EPO's structure (Boissel et al., 1993; Wen et al., 1994). Amino acids predicted to
*comprise the alpha helices A-D in EPO are shown in bold-faced type in Figure 3. Human EPO contains
*four cysteine residues. The disulfide assignments are Cys7 to Cys161 and Cys29 to Cys33.
*   Amino acids in EPO important for receptor binding have been identified through mutagenesis
*experiments and reside primarily in presumptive helices A, C and D (Boissel et al., 1993; Wen et al.,
*1994; Matthews et al., 1996). An important finding relevant to this proposal is that fusion of a six amino
*acid poly-histidine tag to the C-terminus of EPO does not interfere with EPO bioactivity (Boissel et al.,
*1993). This result suggests that other C-terminal fusions, such as those proposed in this grant
*application, also will be active.
*   Only a single cell surface receptor for EPO has been identified (D'Andrea et al., 1989). It is believed
*that EPO dimerizes its receptor in a manner similar to the same way GH dimerizes its receptor
*(Cunningham et al., 1991; de Vos et al., 1992; Matthew's et al., 1996).

Commercial opportunity

*   Recombinant GH, G-CSF and EPO are three of the top five selling protein pharmaceuticals in the
*world, with combined world-wide sales exceeding $4 billion in 1996. Recombinant human GH is used to

*treat short stature and recently received FDA approval for treating cachexia in AIDS patients.
*Recombinant human G-CSF and EPO are used to treat neutropenia and anemia, respectively. EPO
*recently received FDA approval for stimulating red blood cell formation in patients undergoing certain
*types of elective surgeries. Recombinant GH and G-CSF are currently administered by daily subcutaneous
*injection; whereas recombinant EPO is administered by thrice weekly intravenous (dialysis patients) or
*subcutaneous (non-dialysis patients) injections. Novel GH, G-CSF and EPO analogues with longer _in vivo_
*half-lives will allow the same amount of recombinant protein to be administered less frequently. Based
*upon pharmacokinetic studies with other IgG fusion proteins, it should be possible to administer GH-, G-
*CSF- and EPO-IgG fusion proteins once every two to four weeks and maintain effective circulating doses.
*Less frequent dosing should reduce the amount of protein used by patients by more than an order of
*magnitude, with cost savings potentially measured in the hundreds of millions of dollars per year. _In vivo_
*effectiveness of the proteins might be improved because circulating levels of the proteins will be more
*constant. The need for less frequent injections, coupled with improved efficacy, will increase patient
*compliance and quality of life.

*D. Experimental Procedures and Methods

*Cloning of cDNAs encoding GH, G-CSF and EPO

*   A cDNA encoding GH will be amplified by PCR from commercially available single-stranded cDNA
*prepared from human pituitaries (available from CLONTECH, Inc.). A cDNA encoding human G-CSF
*will be purchased from R&D Systems or amplified using PCR from mRNA isolated from human
*carcinoma cell lines such as U87-MG (available from the American Type Culture Collection) known to
*express G-CSF constitutively (Nagata, 1994). A cDNA encoding EPO will be cloned using PCR from
*single-stranded cDNA prepared from human adult kidney or fetal kidney or liver (available from
*CLONTECH, Inc.). PCR primers will be designed based upon the known sequences of GH, G-CSF and
*EPO. Alternatively, synthetic cDNAs encoding full-length GH, G-CSF and EPO will be assembled from
*overlapping oligonucleotides. All clones will be verified by sequencing. The cDNAs will be designed to
*include the N-terminal signal sequences required for secretion of the proteins from the cell. The cDNAs
*also will be designed to delete the termination codon and add an in-frame unique restriction site to
*facilitate joining to DNA sequences encoding the IgG1 C_H or Fc domains. The latter changes will be
*included in the reverse primers used for PCR amplification or in oligonucleotides used to assemble the
*genes. The forward primers will include an optimized Kozak sequence (GCC(A/G)CC<u>ATG</u>G), where the
*underlined ATG is the initiator methionine of the protein) for efficient translation of the proteins in
*mammalian cells (Kozak, 1991).

*Cloning of human IgG1 C_H and Fc domains

*   The Fc and C_H domains of human IgG1 will be cloned using PCR to amplify the appropriate sequences
*from single-stranded human leukocyte cDNA (available from CLONTECH, Inc.). PCR primers will be
*based upon the known sequence of IgG1 DNA (Ellison et al., 1982). The fusion points will be serine at
*position 1 of the CH1 domain and aspartic acid at the beginning of the Fc domain (Ellison et al., 1982).
*Convenient restriction enzyme sites will be incorporated into the PCR primers to facilitate subcloning into
*plasmids and fusion protein construction.

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*Creation of fusion proteins
*   GH-, G-CSF- and EPO- IgG fusion proteins will be assembled in plasmid pUC19, sequenced and
*subcloned into the mammalian cell expression vector pcDNA3.1, available from Invitrogen, Inc.
*pcDNA3.1 can be used for both transient transfection and stable transformation of a variety of
*mammalian cells. The plasmid contains a polylinker for cloning target genes downstream of the strong
*cytomegalovirus promoter, an SV40 origin of replication for high copy number replication in COS cells
*and selectible markers for growth in bacteria (ampicillin resistance) and mammalian cells (G418
*resistance). Plasmid DNAs will be isolated using commercially available kits (e.g., Qiagen, Inc.) and used
*to transfect monkey COS cells in vitro. COS cells will be plated in 10 cm diameter tissue culture dishes
*and transfected the next day with appropriate plasmids using well established procedures (Bebbington,
*1996; Linsley et al., 1991b). Following a 24 hour grow-out in serum-containing media, the cells will be
*washed extensively to remove serum (which could interfere with purification of the IgG fusions by affinity
*chromatography) and grown for an additional 24-72 hours in serum-free media. Conditioned media will
*be collected, concentrated and passed through a Protein A affinity column to purify the IgG fusion
*proteins. Human IgG1 (through the heavy chain constant region) binds tightly to Protein A whereas
*contaminating, residual bovine IgGs, which may be present due to use of bovine serum for initial cell
*growth, bind poorly to this resin (Pierce Immunochemical Reagents Catalogue). Bound proteins will be
*eluted from the column with low pH buffer, immediately neutralized with Tris base and dialyzed. If
*needed, the IgG fusion proteins will be purified further using other chromatographic methods such as ion-
*exchange, hydrophobic interaction or size-exclusion chromatography. Protein concentrations will be
*determined using commercially available protein assay kits (available from Bio-Rad Laboratories).
*Typical yields of other IgG fusion proteins isolated from transfected COS cells are in the range of 1
*mg/liter of conditioned media (Linsley et al., 1991a).
*   If mammalian cell expression of the fusion proteins is not successful, we will express the fusion
*proteins in insect cells as secreted proteins. cDNAs encoding the fusion proteins will be cloned into
*commercially available vectors, e.g., pVL1392 from Invitrogen, Inc. and used to infect insect cells.
*Initially, we will attempt expression of the fusion proteins using the naturally-occurring human signal
*sequences since human signal sequences typically function in insect cells. If we find that one or more of
*the fusion proteins is not secreted efficiently, we will subclone the fusion protein into the expression
*plasmid pMELBAC (available from Invitrogen), which contains a signal sequence from the Honeybee
*mellitin protein and has been used to secrete mammalian proteins from insect cells. We will construct the
*appropriate expression plasmids and then contract the insect cell expression work with the University of
*Colorado Health Sciences Center Core Insect Cell Expression laboratory, which performs this work on a
*fee for service basis. Insect cells will be infected with recombinant viruses in media containing serum,
*grown for 24 hours, washed and grown for several days in serum-free media. Aliquots of the conditioned
*media will be collected on a daily basis and analyzed for the presence of the secreted proteins by SDS-
*PAGE, followed by Western blots using appropriate antisera. Alternatively, secretion of the fusion
*proteins will be detected and quantitated using ELISA assays specific for GH, G-CSF and EPO (R&D
*Systems, Diagnostic Systems Laboratories). Fusion proteins will be purified from conditioned media
*using Protein A affinity chromatography, as described above. Bound proteins will be released from the
*columns using low pH buffer, immediately neutralized and dialyzed. If needed, the proteins will be
*purified further using size exclusion and ion-exchange column chromatography procedures.

*Physical characterization of IgG fusion proteins
*   The IgG fusion proteins will be characterized by SDS-PAGE in the presence and absence of a
*disulfide-reducing agent to determine their relative molecular masses and to determine whether they exist
*as disulfide-linked dimers. Based on studies with other IgG fusion proteins, we expect the proteins to
*exist as disulfide-linked dimers. Dimerization occurs through cysteine residues in the IgG1 heavy chain

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*Hinge region, which is present in all the constructs. The apparent molecular masses of the proteins also
*will be determined by size-exclusion chromatography.

*In vitro evaluation of EPO-IgG fusion proteins
*   EPO-IgG fusion proteins will be tested in cell proliferation assays using the EPO-responsive cell lines
*UT7-epo (Wen et al., 1994) or TF1 (available from the American Type Culture Collection) to measure
*specific activities. Dr. H.F. Bunn (Brigham and Women's Hospital, Boston, MA) has agreed to provide us
*with the UT7-epo cell line. Cells will be plated in 96-well microtiter plates with serial 3-fold dilutions of
*EPO, EPO-IgG fusion proteins, human IgG1 or buffer. Assays will be performed in triplicate. After 1-3
*days in culture, the cells will be washed, incubated for 4 h with ^{3}H-thymidine and harvested for
*determination of incorporated radioactivity by scintillation counting. Alternatively, we will develop a
*nonradioactive dye uptake assay employing MTT (Sigma) to measure cell proliferation/viability. For
8these assays, cells will be grown for 2-4 days in the presence of EPO, EPO-IgG fusion proteins, human
*IgG1 or buffer before treatment with MTT. The cells will be solubilized, incubated overnight to allow
*color formation and absorbance of the plates read the next day using a microtiter plate reader. The EC_{50}
*will be determined for each protein. Assays will be performed at least three times for each protein and
*with triplicate wells for each data point. EC_{50} comparisons will be used to compare the relative potencies
*of EPO and the EPO-IgG fusion proteins. Fusion proteins displaying similar optimal levels of stimulation
*(90% or more) and EC_{50} values comparable to EPO (within 2-fold) will be considered for further study.

*Phase II - In vivo evaluation of GH-, G-CSF- and EPO-IgG fusion protein candidates.
*   We expect that the Phase I experiments described above will allow us to determine whether the GH-, G-
*CSF- and EPO- IgG fusion proteins possess bioactivities comparable to that of GH, G-CSF and EPO in
*in vitro assays. Demonstration that at least one of the IgG fusion proteins possesses wild type in vitro
*bioactivity will be the criterion used for successful completion of Phase I. Fusion proteins that retain full
*activity in in vitro assays will be candidates for further development. If both the C_H and Fc IgG fusion
*proteins posess full activity, we will analyze both during Phase II to determine which performs best in
*animal disease models.

*The following experiments will be performed during the Phase II portion of the grant.
Construction of stable mammalian cell lines expressing the IgG fusion proteins
*   In order to obtain the large amounts of protein required for animal experiments we will need to develop
*larger scale processes for expression and purification of the IgG fusion proteins using stably transformed
*mammalian cells. We expect the purification scheme will be similar to the one developed for small-scale
*production of the fusion proteins. For stable expression in mammalian cells, pcDNA3.1 plasmids
*encoding the IgG fusion proteins will be used to stably transform DHFR⁻ CHO or rodent NS0 myeloma
*cells using G418 antibiotic resistance to select for cells expressing stably-integrated plasmid DNA. A
*plasmid encoding glutamine synthetase or dihydrofolate reductase (DHFR) will be co-transfected with the
*pcDNA3.1 IgG fusion protein plasmids to allow for gene amplification at a later time to increase protein
*production levels. Following selection of single cells by limited dilution cloning, sublines will be
*developed and screened for GH-, G-CSF- or EPO-IgG expression using GH, G-CSF, EPO and IgG
*ELISA assays (R&D Systems, Diagnostic Systems Laboratories). Increases levels of protein production
*can be achieved by selection using increasing concentrations of methotrexate (DHFR⁻ CHO cells) or

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*methionine sulphoximine for NS0 cells) in the growth media. Large scale mammalian cell culture work
*will be contracted to the Colorado Bioprocess Center at Colorado State University, which performs this
*service on a fee for service basis.

*Expression and purification of control proteins - GH, G-CSF and EPO
*   Large amounts (tens of milligrams) of non-fused GH, G-CSF and EPO will be required as controls for
*the animal experiments and will have to be manufactured as well. Recombinant human GH and G-CSF
*for human use are manufactured in bacteria, whereas recombinant EPO needs to be manufactured using
*mammalian cells. GH will be expressed in E. coli using the STII or OmpA signal sequences to secrete the
*mature protein into the periplasmic space (Hsiung et al., 1986; Chang et al., 1987). Secreted GH is
*properly folded and biologically active. Following osmotic shock, the secreted protein will be purified by
*conventional column chromatography methods. G-CSF will be produced as an intracellular protein in E.
*coli. The recombinant protein is insoluble, but can be refolded into an active form using well known
*procedures (Souza et al., 1986). Recombinant EPO will need to be manufactured using mammalian CHO
*cells to ensure proper glycosylation (Delorme et al., 1982). Stable cell lines secreting EPO will be
*prepared as described above for the fusion proteins. Recombinant EPO will be epitope tagged (using a
*(His)$_6$ or FLAG (Kodak) tag) at its C-terminus to facilitate purification by affinity chromatography.

*Pharmacokinetic experiments with IgG fusion proteins
*   We will collaborate with researchers at the Office of Laboratory Animal Resources at the University of
*Colorado Health Sciences Center to perform pharmacokinetic studies of the fusion proteins to determine
*to what fusion of the proteins to the Fc or C$_H$ domains of human IgG1 extends the _in vivo_ half lives of the
*proteins. The Office of Laboratory Animal Resources is an accredited animal research facility. These
*data will guide us in designing animal experiments to determine dosing regimens to compare the
*efficiencies of IgG fusion proteins to the natural proteins. Pairs of rats will receive an intravenous bolus
*injection of each protein and circulating levels of the proteins will be measured over the course of 24 h.
*Protein levels will be quantitated using commercially available human ELISA kits for GH, G-CSF and
*EPO (R&D Systems and Diagnostoc Systems Laboratories). Additional experiments will be performed
*using the subcutaneous route of administration. Similar experiments will be performed with the non-
*fusion proteins. We expect to find that (1) fusion of the proteins to the Fc or C$_H$ domains of human IgG
*significantly extends the circulating half-lives of GH, G-CSF and EPO relative to the natural proteins and
*(2) fusion to the larger C$_H$ domain of IgG1 extends the circulating half-life more than fusion to the smaller
*Fc domain.

*Animal disease models - general considerations
*   We will compare the relative efficacies of the C$_H$ and Fc IgG fusion proteins in appropriate animal
*disease models, as described below. Appropriate dosing schedules will be determined for each fusion
*protein. We expect to find that the larger fusion protein molecule increases the circulating half-life greater
*than the smaller fusion protein molecule and will require less frequent dosing. However, large proteins
*may have reduced volumes of distribution _in vivo_; thus it is possible the larger fusion proteins may limit
*bioavailability, reducing its efficacy. Animal disease models will allow us to determine if this is the case.
*Once the optimum dosing schedule and fusion protein size are determined, we will compare the efficacies
*of the fusion proteins to each other and to the non-fused proteins in the animal models. We expect to find
*that the IgG fusion proteins produce results equal or superior to the non-fused proteins, but can be given
*less frequently. We expect to find that the IgG fusion proteins are more efficacious than the non-fusion
*proteins when both are administered using the less frequent dosing schedules.

*Animal anemia models to test EPO-IgG development candidates

*   In vivo bioactivities of the EPO-IgG fusion proteins will be tested using the artificial polycythemia or
*starved rodent models (Cotes and Bangham., 1961; Goldwasser and Gross., 1975). In the starved rodent
*model, rats are deprived of food on day one and treated with test samples on days two and three. On day
*four, rats receive an injection of radioactive iron-59. Approximately 18h later, rats are anesthetized and
*blood samples drawn. The percent conversion of labeled iron into red blood cells is then determined. In
*the artificial polycythemia model, mice are maintained in a closed tank and exposed for several days to
*hypobaric air. The animals are then brought to normal air pressure. Red blood cell formation is
*suppressed for several days. On day four or six after return to normal air pressure, mice are injected with
*EPO or saline. Mice receive one injection per day for one to two days.   One day later the animals receive
*an intravenous injection of labeled iron-59. The mice are euthanized 20 h later and the amount of labeled
*iron incorporated into red blood cells determined. EPO stimulates red blood cell formation in both

*models as measured by a dose-dependent increase in labeled iron incorporated into red blood cells. In
*both models we will study different dosing regimens and different times of injections to determine if EPO-
*IgG is more potent and produces longer acting effects than natural EPO.
*   We will contract with an accredited commercial animal testing facility or an academic laboratory to
*perform these experiments. We also will identify an academic consultant with experience with these
*animal models to help us in the performance of the experiments.

*Research summary:
*   Successful completion of the Phase I and Phase II studies outlined in this proposal will allow the
*creation of long-acting versions of GH, G-CSF and EPO for use in treating short stature, cachexia,
*neutropenia and anemia. These studies will provide valuable basic structure/function information about
*three of the most important human therapeutic proteins discovered to date. In particular, these studies
*should provide important data concerning the role of the C-terminus of the proteins in binding to cell
*surface receptors and activating intracellular signaling pathways. More generally, knowledge gained from
*these studies will expand the potential number of cytokines and growth factors for which IgG fusion
*technology has been assessed. Successful development of fully active, long-lived GH, G-CSF- or EPO-
*IgG fusion proteins will stimulate creation of additional cytokine/growth factor-IgG fusion proteins using
*the same technology, particularly with other members of the GH supergene family. These long-lived
*proteins will find applications in treating a number of chronic disease indications, including endocrine,
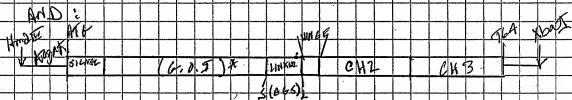*hematopoietic and inflammatory disorders and cancer.

EXHIBIT C

OCTOBER 1, 1998

CONSTRUCT FUSIONS of (GH, EPO, GCSF)

TO (N/Ag) CH1 OF IgG1 + IgG4 +

(N/Ag) HINGE REGION IgG1 + IgG4

IN GENERAL :

*G.O.I = hu GH, hu EPO, hu G-CSF
(Himedin 160a cost)

Clone FUSIONS in pCDNA3.1 (+) (INVITROGEN)
FOR EXPRESSION

① PCR IgG1 + IgG4 CH1 → CH3 + [hinge → CH3]
as Bam + Xba clone + sequence in pCDNA3.1

② PCR EACH G.O.I AS HindIII + BamHI, clone
and sequence in pCDNA3.1 (+)

③ Reject + COMBINE G.O.I's w Igg segment
THROUGH BamHI SITE-

Project No. _____
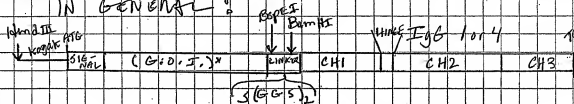Book No. _____
TITLE **IGG FUSIONS w GH, EPO, GCSF**

Page No. _____

OCTOBER 1, 1998

CONSTRUCT FUSIONS OF (GH, EPO, GCSF) C-term

to (AND) CH1 OF IgG1 + IgG4 +

(AND) HINGE REGION IgG + IgG4

IN GENERAL:

*G.O.I - hu GH, hu EPO, hu G-CSF
(HindIII both end)

Clone FUSIONS in pCDNA3.1 (+) (INVITROGEN)
FOR EXPRESSION

① PCR IgG1 + IgG4 [CH1 → CH3] + [Hinge → CH3]
as Bam - Xba clone + sequence in pCDNA3.1 (+)

② PCR EACH G.O.I AS HindIII - BamHI, clone
and sequence in pCDNA3.1 (+)

③ Resect + combine G.O.I's w IgG segments
through BamHI site -

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Witnessed & Understood by me, _____ Date 10/17/88
Invented by [signature]
Recorded by
Date 10/19/98

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OCTOBER 1, 1998

Bam
```
GGT GCC TCC ACC AAG GGC CCA TCG GTC TTC CCC CTG GCA CCC TCC TCC AAG AGC ACC
       CCG AGG TGG TTC CCG GGT AGC CAG AAG GGG GAC CGT GGG AGG AGG TTC TCG TGG
    A   S   T   K   G   P   S   V   F   P   L   A   P   S   S   K   S   T
```

```
TCT GGG GGC ACA GCG GCC CTG GGC TGC CTG GTC AAG GAC TAC TTC CCC GAA CCG
AGA CCC CCG TGT CGC CGG GAC CCG ACG GAC CAG TTC CTG ATG AAG GGG CTT GGC
  S   G   G   T   A   A   L   G   C   L   V   K   D   Y   F   F   E   P
```

```
GTG ACG GTG TCG TGG AAC TCA GGC GCC CTG ACC AGC GGC GTG CAC ACC TTC CCG
CAC TGC CAC AGC ACC TTG AGT CCG CGG GAC GTT TCG CCG ACA GTG TGG AAG GGC
  V   T   V   S   W   N   S   G   A   L   T   S   G   V   H   T   F   P
```

```
GCT GTC CTA CAG TCC TCA GGA CTC TAC TCC CTC AGC AGC GTG GTG ACC GTG CCC
CGA CAG GAT GTC AGG AGT CCT GAG ATG AGG GAG TCG TCG CAC CAC TGG CAC GGG
  A   V   L   Q   S   S   G   L   Y   S   L   S   S   V   V   T   V   P
```

```
TCC AGC AGC TTG GGC ACC CAG ACC TAC ATC TGC AAC GTG AAT CAC AAG CCC AGC
AGG TCG TCG AAC CCG TGG GTC TGG ATG TAG ACG TTG CAC TTA GTG TTC GGG TCG
  S   S   S   L   G   T   Q   T   Y   I   C   N   V   N   H   K   P   S
```

```
AAC ACC AAG GTG GAC AAG AAA GTT GAG CCC AAA TCT TGT GAC AAA ACT CAC ACA
TTG TGG TTC CAC CTG TTC TTT CAA CTC GGG TTT AGA ACA CTG TTT TGA GTG TGT
  N   T   K   V   D   K   K   V   E   P   K   S   C   D   K   T   H   T
```

```
TGC CCA CCG TGC CCA GCA CCT GAA CTC CTG GGG GGA CCG TCA GTC TTC CTC TTC
ACG GGT GGC ACG GGT CGT GGA CTT GAG GAC CCC CCT GGC AGT CAG AAG GAG AAG
  C   P   P   C   P   A   P   E   L   L   G   G   P   S   V   F   L   F
```

```
CCC CCA AAA CCC AAG GAC ACC CTC ATG ATC TCC CGG ACC CCT GAG GTC ACA TGC
GGG GGT TTT GGG TTC CTG TGG GAG TAC TAG AGG GCC TGG GGA CTC CAG TGT ACG
  P   P   K   P   K   D   N   L   M   I   S   R   T   P   E   V   T   C
```

```
GTG GTG GTG GAC GTG AGC CAC GAA GAC CCT GAG GTC AAG TTC AAC TGG TAC GTG
CAC CAC CAC CTG CAC TCG GTG CTT CTG GGA CTC CAG TTC AAG TTG ACC TAG ACA
  V   V   V   D   V   S   H   E   D   P   E   V   K   F   N   W   Y   V
```

```
GAC GGC GTG GAG GTG CAT AAT GCC AAG ACA AAG CCG CGG GAG GAG CAG TAC AAC
CTG CCG CAC CTC CAC GTA TTA CGG TTC TGT TTC GGC GCC CTC CTC GTC ATG TTG
  D   G   V   E   V   H   N   A   K   T   K   P   R   E   E   Q   Y   N
```

```
AGC ACG TAC CGT GTG GTC AGC GTC CTC ACC GTC CTG CAC CAG GAC TGG CTG AAT
TCG TGC ATG GCA CAC CAG TCG CAG GAG TGG CAG GAC GTG CTG GTC CTG ACC GAC TTA
  S   T   Y   R   V   V   S   V   L   T   V   L   H   Q   D   W   L   N
```

```
GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA GCC CTC CCA GCC CCC ATC GAG
CCG TTC CTC ATG TTC ACG TTC CAG AGG TTG TTT CGG GAG GGT CGG GGG TAG CTC
  G   K   E   Y   K   C   K   V   S   N   K   A   L   P   A   P   I   E
```

```
AAA ACC ATC TCC AAA GCC AAA GGG CAG CCC CGA GAA CCA CAG GTG TAC ACC CTG
TTT TGG TAG AGG TTT CGG TTT CCC GTC GGG GCT CTT GGT GTC CAC TAG TGG GAC
  K   T   I   S   K   A   K   G   Q   P   R   E   P   Q   V   Y   T   L
```

```
CCC CCA TCC CGG GAT GAG CTG ACC AAG AAC CAG GTC AGC CTG ACC TGC CTG GTC
GGG GGT AGG GCC CTA CTC GAC TGG TTC TTG GTC CAG TCG GAC TGG ACG GAC CAG
  P   P   S   R   D   E   L   T   K   N   Q   V   S   L   T   C   L   V
```

```
AAA GGC TTC TAT CCC AGC GAC ATC GCC GTG GAG TGG GAG AGC AAT GGG CAG CCG
TTT CCG AAG ATA GGG TCG CTG TAG CGG CAC CTC ACC CTC TCG TTA CCC GTC GGC
  K   G   F   Y   P   S   D   I   A   V   E   W   E   S   N   G   Q   P
```

```
GAG AAC AAC TAC AAG ACC ACG CCT CCC GTG CTG GAC TCC GAC GGC TCC TTC TTC
CTC TTG TTG ATG TTC TGG TGC GGA GGG CAC GAC CTG AGG CTG CCG AGG AAG AAG
  E   N   N   Y   K   T   T   P   P   V   L   D   S   D   G   S   F   F
```

```
CTC TAC AGC AAG CTC ACC GTG GAC AAG AGC AGG TGG CAG GAG GGG AAC GTC TTC
GAG ATG TCG TTC GAG TGG CAC CTG TTC TCG TCC ACC GTC GTC CTC CCC TTG CAG AAG
  L   Y   S   K   L   T   V   D   K   S   R   W   Q   Q   G   N   V   F
```

```
TCA TGC TCC GTG ATG CAT GAG GCT CTG CAC AAC CAC TAC ACG CAG AAG AGC CTC
AGT ACA AGG CAC TAC GTA CTC CGA CGA GTG GTG ATG TGC GTC TTC TCG GAG
  S   C   A   V   M   H   E   A   L   H   N   H   Y   T   Q   K   S   L
```

```
TCC CTG TCT CCG GGT AAA TGA gtgcg acggccggca agcccccgct ccccgggctc
AGG GAC AGA GGC CCA TTT ACT cacgc tgccggccgt tcggggcga gggggcccggag
  S   L   S   P   G   K   *
```

```
tcgcggtcgc acgaggatgc ttggcacgta ccccgtgtac atacttcccg gggcccagc
aggcccagcg tcctcctacg aacgtgcat ggggcacgtg tatgaaggc ccgcgggtcg
```

IgG1
SEQUENCE
+ POSITION
OF RELE-
VANT
OLIGOS

OLIGO
SEQUENCES
GIVEN
p 147

GCT TCC ACC AAG GGC CCA TCC GTC TTC CCC CTG GCG CCC TGC TCC AGG AGC ACC
CGA AGG TGG TTC CCG GGT AGG CAG AAG GGG GAC CGC GGG ACG AGG TCC TCG TGG
A   S   T   K   G   P   S   V   F   P   L   A   P   S   S   K   S   T

TCC GAG AGC ACA GCC GCC CTG GGC TGC CTG GTC AAG GAC TAC TTC CCC GAA CCG
AGG CTC TCG TGT CGG CGG GAC CCG ACG GAC CAG TTC CTG ATG AAG GGG CTT GGC
S   E   S   T   A   A   L   G   C   L   V   K   D   Y   F   P   E   P

GTG ACG GTG TCG TGG AAC TCA GGC GCC CTG ACC AGC GGC GTG CAC ACC TTC CCG
ACA TGC CAC AGC ACC TTG AGT CCG CGG GAC TGG TCG CCG CAC GTG TGG AAG GGC
V   T   V   S   W   N   S   G   A   L   T   S   G   V   H   T   F   P

GCT GTC CTA CAG TCC TCA GGA CTC TAC TCC CTC AGC AGC GTG GTG ACC GTG CCC
CGA CAG GAT GTC AGG AGT CCT GAG ATG AGG GAG TCG TCG CAC CAC TGG CAC GGG
A   V   L   Q   S   S   G   L   Y   S   L   S   S   V   V   T   V   P

TCC AGC AGC TTG GGC ACG AAG ACC TAC ACC TGC AAC GTA GAT CAC AAG CCC AGC
AGG TCG TCG AAC CCG TGC TTC TGG ATG TGG ACG TTG CAT CTA GTG TTC GGG TCG
S   S   S   L   G   T   K   T   Y   T   C   N   V   D   H   K   P   S

AAC ACC AAG GTG GAC AAG AGA GTT GAG TCC AAA TAT GGT CCC CCA TGC CCA
TTG TGG TTC CAC CTG TTC TCT CAA CTC AGG TTT ATA CCA GGG GGT ACG GGT
N   T   K   V   D   K   R   V   E   S   K   Y   G   P   P   C   P

TCA TGC CCA GCA CCT GAG TTC CTG GGG GGA CCA TCA GTC TTC CTG TTC
AGT ACG GGT CGT GGA CTC AAG GAC CCC CCT GGT AGT CAG AGA GAC AAG
S   C   P   A   P   E   F   L   G   G   P   S   V   F   L   F

CCC CCA AAA CCC AAG GAC AAG TCT CTC ATA ATC TCC CGG ACC CCT GAG GTC ACG TGC
GGG GGT TTT GGG TTC CTG TTC AGA GAG TAT TAG AGG GCC TGG GGA CTC CAG TGC ACG
P   P   K   P   K   D   T   L   M   I   S   R   T   P   E   V   T   C

GTG GTG GTG GAC GTG AGC CAG GAA GAC CCC GAG GTC CAG TTC AAC TGG TAC GTG
CAC CAC CAC CTG CAC TCG GTC CTT CTG GGG CTC CAG GTC AAG TTG ACC TAG ACA
V   V   V   D   V   S   Q   E   D   P   E   V   Q   F   N   W   Y   V

GAT GGC GTG GAG GTG CAT AAT GCC AAG ACA AAG CCG CGG GAG GAG CAG TTC AAC
CTA CCG CAC CTC CAC GTA TTA CGG TTC TGT TTC GGC GCC CTC CTC GTC AAG TTG
D   G   V   E   V   H   N   A   K   T   K   P   R   E   E   Q   F   N

AGC ACG TAC CGT GTG GTC AGC GTC CTC ACC GTC CTG CAC CAG GAC TGG CTG AAC
TCG TGC ATG GCA CAC CAG TCG CAG GAG TGG CAG GAC GTG GTC CTG ACC GAC TTG
S   T   Y   R   V   V   S   V   L   T   V   L   H   Q   D   W   L   N

GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA GGC CTC CCG TCC TCC ATC AAG
CCG TTC CTC ATG TTC ACG TTC CAG AGG TTG TTT CCG GAG GGC AGG AGG TAG CTC
G   K   E   Y   K   C   K   V   S   N   K   G   L   P   S   S   I   E

AAA ACC ATC TCC AAA GCC AAA GGG CAG CCC CGA GAG CCA CAG GTG TAC ACC CTG
TTT TGG TAG AGG TTT CGG TTT CCC GTC GGG GCT CTC GGT GTC CAC TAG TGG GAC
K   T   I   S   K   A   K   G   Q   P   R   E   P   Q   V   Y   T   L

CCC CCA TCC CAG GAG GAG ATG ACC AAG AAC CAG GTC AGC CTG ACC TGC CTG GTC
GGG GGT AGG GTC CTC CTC TAC TGG TTC TTG GTC CAG TCG GAC TGG ACG GAC CAG
P   P   S   Q   E   E   M   T   K   N   Q   V   S   L   T   C   L   V

AAA GGC TTC TAC CCC AGC GAC ATC GCC GTG GAG TGG GAG AGC AAT GGG CAG CCG
TTT CCG AAG ATG GGG TCG CTG TAG CGG CAC CTC ACC CTC TCG TTA CCC GTC GGC
K   G   F   Y   P   S   D   I   A   V   E   W   E   S   N   G   Q   P

GAG AAC AAC TAC AAG ACC ACG CCT CCC GTG CTG GAC TCC GAC GGC TCC TTC TTC
CTC TTG TTG ATG TTC TGG TGC GGA GGG CAC GAC CTG AGG CTG CCG AGG AAG AAG
E   N   N   Y   K   T   T   P   P   V   L   D   S   D   G   S   F   F

CTC TAC AGC AGG CTA ACC GTG GAC AAG AGC AGG TGG CAG GAG GGG AAT GTC TTC
GAG TAG TCG TCC GAT TGG CAC CTG TTC TCG TCC ACG TCC TTC CCC TTA CAG AAG
L   Y   S   R   L   T   V   D   K   S   R   W   Q   E   G   N   V   F

TCA TGC TCC GTG ATG CAT GAG GCT CTG CAC AAC CAC TAC ACA CAG AAG AGC CTC
AGT ACG AGG CAC TAC GTA CTC CGA GAC GTG TTG GTG ATG TGT GTC TTC TCG GAG
S   C   S   V   M   H   E   A   L   H   N   H   Y   T   Q   K   S   L

TCC CTG TCT CTG GGT AAA TGA gtgccagggccggcaagccccgtctccccgggctctcgggg
AGG GAC AGA GAC CCA TTT ACT cacggtccccggcacgttcggggggtcgaggggcccgagagcccc
S   L   S   L   G   K   *

5bp   act cct aag aac cgt gca tgg aga tct cgc

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Ig G 1/4   OLL 60S

OCTOBER 1, 1998

BB81 (IgG1/4 CH1 forward)   5>CGC GGA TCC GGT GGC TCA GCC TCC
     ACC AAG GGC CCA TC>3

BB82 (IgG1/4 3'UTR rev) 5> CGC TCT AGA GGT ACG TGC CAA
     GCA TCC TCG >3

BB83 (IgG1 HINGE forward) 5>CGC GGA TCC GGT GGC TCA GAG
     CCC AAA TCT TGT GAC AAA ACT>3

BB84 (IgG4 HINGE forward) 5> CGC GGA TCC GGT GGC TCA GAG
     TCC AAA TAT GGT CCC CCA TGC>3

BB85 (IgG4 HINGE forward) 5> TCC AAA TAT GGT CCC CCA TGC
     CCA TCA >3

BB86 (IgG4 HINGE reverse)   5> TGG GCA TGA TGG GCA TGG
     GGG ACC ATA >3

BB87 (GH IGGFUS forward) 5> CGC AAG CTT GCC ACC ATG GCT
     ACA GGC TCC CGG ACG >3

BB102 (IGG1/4 SEQ forward in CH2) 5>TTC CCC CCA AAA CCC AAG
     GAC AC>3

BB103 (IGG1/4 SEQ forward in CH3) 5>CAG GTC AGC CTG ACC TGC
     CTG GTC>3

BB104 (IGG1/4 SEQ reverse in CH2, complement of BB102) 5>GT
     GTC CTT GGG TTT TGG GGG GAA>3

BB105 (IGG1/4 SEQ reverse in CH3, complement of BB103)
     5>GAC CAG GCA GGT CAG GCT GAC CTG>3

SET UP PCRS FOR :

IgG1 "CH1" FULL LENGTH CHI, Hinge, CH2, 3

& "Hinge" Hinge, CH2 CH3

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OKTOBER
1998

PCRS: TEMPLATE = 1µl, 1 ng CLONTECH HPB leukocyte
cDNA, CAT# 7182-1

"CH1"   #1   "Hinge" #2
1 µl   TEMPLATE   1 µl   TEMPLATE
10 µl   10x PCR Buffer (PROMEGA)   10 µl   10x PCR Buffer (RRA)
10 µl   10x dNTPs   10 µl   10x dNTPs
6 µl   25 mM MgCl₂   6 µl   25 mM MgCl₂
2.5 µl BB81 (10 pmol/µl)   2.5 µl BB83 (10 pmol/µl)
2.5 µl BB82 (10 pmol/µl)   2.5 µl BB82   "
0.3 µl Taq Pol (PM)   0.3 µl Taq Pol (PM)
0.1 µl Pfu Pol (STRAT)   0.1 µl Pfu Pol (STRAT)
67.6 µl H₂O   67.6 µl H₂O

MAKE 2x EACH Rxn → Σ 200 µl D₁+₂

SPLIT EACH → 50 µl x3 + ANNEAL @ 3 temps
       100 µl → 50 µl x2   D₁/₂

CYCLE: 96°C   3 MIN
        95°C   1 MIN
   33   58°(oc 66°) 1 MIN   α 2:35 → 4:45
 cycles 72°C   1½ MIN
        4°C   HOLD

                                      125 V
                                      START - 4:50
RUN OUT: 1.2% Ag GR, 1X TBE   STOP - 5:30

1 λ/H + ΦX |
2 -   [black rectangle]   LOOKS GOOD -
3 PCR-1 (58°) 5/60 µl
4   "   (66°) "   SIZES ALL
5 -   CONSISTENT WITH
6 PCR 2 (58°) 5/50   EXPECTED -
7   "   (66°) "
8   BOTH ANNEALING
9 λ/H + Φx   TEMPS SEEM TO
                                                     WORK!

Witnessed & Understood by me,   Date   Invented by
        Joe C   10/19/98
                                               Recorded by   Date
                                                                     10/19/98 F

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OCTOBER 2, 1998

CLEAN UP PCR-s : Pool Both temp Rxns —

    Clean up w/ Qia spin PCR clean up — Elute 50 µl EB

    Go to digests ° : BamHI + XbaI

    PCR ①   PCR ②   pCDNA3.1 (+)
                                               (9/23/98)
    ~45 µl PCR ①   ~45 µl PCR ②   7 µl QSP ~ 2 µg
    40 µl 1k H₂O   40 µl H₂O   81 µl H₂O
    10 µl NEB2 (10X)   10 µl NEB2 (10X)   10 µl 10x NEB2
    1 µl 100x BSA   1 µl 100x BSA   1 µl 100 > 1 BSA
    2 µl BamHI 40u   2 µl BamHI 40u   0.5 µl BamHI 10u
    2 µl XbaI 40u   2 µl XbaI 40u   0.5 µl XbaI 10u

    All @ 37°C ~ 10:40

    @ 12:10   ADD 1 µl CIP (10u) NEB TO pCDNA only

    @ 1:10   — EtOH Precip, wash Pellet w/ 70% EtOH

              AIR DRY ~37°C ~30 min Resus +
                                            run out
    PREP GEL PRGS :   1.2% Az Gel 1X TAE   START ~ 2:15
                                              STOP ~ 3:30
    1   Øx
    2   pCR① x   Bam + Xba   XCISE BANDS
    3   —   Elute in QIAEX II
    4   pCR② x   Bam + Xba
    5   Elute Resin
                                              w/ 25 µl EB
                                              pull 20 µl
                                              STORE @ -20°C
                                              o/n

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Witnessed & Understood by me,   Date   Invented by   Date
                         10/19/98   10/19/98
                                  Recorded by

OCTOBER 2, 1998

pcDNA3.1(+) prep gel: 0.6% Ag 5x 12x TAE

START - 2:15
STOP - 3:30

lanes
1 λ/H
2 pcDNA3.1(+) Nat (1.5?)
3 pcDNA3.1(+) x(Bam Xen + CIP)

well separated
linear vs uncut

but maybe some
degradation of
Bam-Xba vector?

xcise vector band

Elute (Qiagen), Elute resin
with 25 ul H₂O, pull 20 ul, start @ ~ 2:00

10/4 set up ligations: 10/4

Lig ①

2 ul pcDNA3.1 (BXC(P) gp
2 ul pUC19 (B-X) gp
3.5 ul H₂O
2 ul 5x lig Buffer (BRL)
0.5 ul T4 ligase 0.5 units (BRL)

Lig ②

2 ul pcDNA3.1 (BXC P) gp
2 ul pUC② (BX) gp
9.5 ul H₂O
2 ul 5x lig Buffer BRL
0.5 ul T4 Ligase

Lig ③

2 ul pcDNA3.1 (BXC
2 ul H₂O
3.5 ul H₂O
2 ul 5x lig Buffer
0.5 ul T4 Ligase

@ 15°C ON ~ 2:30p 10/4

10/5 pull ligs & go to Tfm p 151

OCTOBER 5, 1998

Transform with lungs 1, 2, 3 (10|4)

T=0 5µl each Hg, 5µl BLANK Φ DNA + 50µl comp DH5α

HOLD ON ICE 10min, → 42°C for 45 sec,

CHILL ON ICE, dil 1/10 W 50Cc, in 37°C ~ 60min

PLATE OUT LB Ap^100 10µl, 100µl each

@ 37°C ~ 5:15p

10/6   PULL PLATES, LOOKS GOOD —

(CHI) Lung ①   10µl ; 11 cfu
                100µl ; ~(100-200)   LOOKS GOOD

(lungs) Lung ②   10µl   ;   ~50
                 100µl   ;   ~500   P(CH⁴ 150µl in cols
         Lung ③   10µl   ;   Φ   from #1, #2
                 100µl   ;   8 cfu   inoc 4-5ml LB Ap^100
                                            o/n 37° — ON
         BLANK   10µl   ;   Φ
                 20µl   ;   Φ   SAVE PLATES @ 4°C

10/7   ONS   up   OK   1·(1→4) 2·(1→4) → SINGLES
                                             ON LB Ap^100
         QSP (2ml/EPP) ×2   elute each 50µl ③, pool in 100µl

         OD's dil 1/100 A₂₆₀=   A₂₆₀   conc   A₂₆₀ conc
                               1-1 .106   0.53 8/2   2-1 .091 0.46 8/µl
                               1-2 .157   0.79 "   2-2 .108 0.54 "
                               1-3 .150   0.75 "   2-3 .128 0.64 "
                               1-4 .117   0.59 "   2-n .084 0.42 "

         cut & run

OCTOBER 8, 1998

Digest + run out to check insert—

	x BmHI			x BmHI + KpnI
10x	~3µl	Q8l	10x	~3µl Q8P
6°	5µl	10x NEB2	50	5µl 10x NEB2
420	42µl	H₂O	410	41µl H₂O
5	0.5µl	BmHI (10u)	5	0.5µl Bm (10u)
			5	0.5µl KpnI (10u)

MAKE up 10x Rxn MIX, ALIQUOT 47µl to TUBE

WITH VARYING AMNT OF PLASMID = 2µg

1-1 :	4µl	2-1 :	4µl	pCDNA3.1(+): 7µl
1-2 :	2.5µl	2-2 :	4µl	
1-3 :	2.5µl	2-3 :	3.3µl	
1-4 :	3.3µl	2-4 :	5µl	

Ave Digest @ 37°C @ 10:00 → 11:30

RUN OUT 10µl / 50µl Digests   0.7% Ag 5K 1X TBE

GEL ①		Gel ②		① 75V	START - 11:4:
LANES	14 + Dx	LANE			STOP - 1:00
1 pCDNA x Bm 10/50	1	X14 + Dx			
2 1-1		2 pCDNA x Bm x Kpn 10/50		
3 1-2		3 1-1			
4 1-3		4 1-2		② 75V	START - 11:55
5 1-4		5 1-3			STOP - 1:00
6 2-1		6 1-4			
7 2-2		7 2-1			
8 2-3		8 2-2			
9 2-4		9 2-3			
10		10 2-4			

PHOTO3   p 167

Witnessed & Understood by me, _____ Date 10/19/98   Invented by _____ Date 10/19/98   Recorded by

LE _____

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OCTOBER 8 1998

ALL LOOK OK, INSERTS CORRECT SIZE,
ONLY ONE INSERT + QUANTITY CONSISTENT
WITH OD —

SEND OFF TO MMR FOR SEQ.   10/8/98

TEMPLATES 10/8/98

#		CONC (ug/ul)	VOL (ul)
QSP 1-1	(10/7/98)	0.53	40
" 1-2	"	0.79	40
" 1-3	"	0.75	40
" 1-4	"	0.59	40
" 2-1	"	0.46	40
" 2-2	"	0.54	40
" 2-3	"	0.64	40
" 2-4	.	0.42	40

To Page No._____

PCR OUT EPO + GH WITH C-TERMINAL SEQS
FOR FUSION VIA RECOMBINATION WITH
IGG CLONES AT LINKER S(G4S)2

GROWTH HORMONE SEQ + OL16-55

h GH w signal

27↓

```
ATGGCTACAGGCTCCCGGACGTCCCTGCTCCTGGCTTTTGGCCTGCTCTGCCTGCCCTGGCTTCAAGAGGGCAGTGCCTTCCCAACCATT
 M  A  T  G  S  R  T  S  L  L  L  A  F  G  L  L  C  L  P  W  L  Q  E  G  S  A  F  P  T  I
CCCTTATCCAGGCTTTTTGACAACGCTATGCTCCGGGCCCATCGTCGCACCAGCTGGCCTTTGACACCTACCAGGAGTTTGAAGAAGCC
 P  L  S  R  L  F  D  N  A  M  L  R  A  H  R  L  H  Q  L  A  F  D  T  Y  Q  E  F  E  E  A
TATATCCCAAAGGACAGAAGTATTCATTCCTGCAGAACCCCCAGACCTCCCTCTGTTTCTCAGAGTCTATTCCGACACCCTCCAACAGG
 Y  I  P  K  E  Q  K  Y  S  F  L  Q  N  P  Q  T  S  L  C  F  S  E  S  I  P  T  P  S  N  R
                                          43↓
GAGGAAACACAACAGAAATCCAACCTAGAGCTGCTCCGCATCTCCCTGCTGCTCATCCAGTCGTGGCTGGAGCCCGTGCAGTTCCTCAGG
 E  E  T  Q  Q  K  S  N  L  E  L  L  R  I  S  L  L  L  I  Q  S  W  L  E  P  V  Q  F  L  R
AGTGTCTTCGCCAACAGCCTGGTGTACGGCGCCTCTGACAGCAACGTCTATGACCTCCTAAAGGACCTAGAGGAAGGCATCCAAACGCTG
 S  V  F  A  N  S  L  V  Y  G  A  S  D  S  N  V  Y  D  L  L  K  D  L  E  E  G  I  Q  T  L
                                          ↑GTNACRCAGTCCAGGRACG q
ATGGGGAGGCTGGAAGATGGCAGCCCCCGGACTGGGCAGATCTTCAAGCAGACCTACAGCAAGTTCGACACAAACTCACACAACGATGAC
 M  G  R  L  E  D  G  S  P  R  T  G  Q  I  F  K  Q  T  Y  S  K  F  D  T  N  S  H  N  D  D
GCACTACTCAAGAACTACGGGCTGCTCTACTGCTTCAGGAAGGACATGGACAAGGTCGAGACATTCCTGCGCATCGTGCAGTGCCGCTCT
 A  L  L  K  N  Y  G  L  L  Y  C  F  R  K  D  M  D  K  V  E  T  F  L  R  I  V  Q  C  R  S
GTGGAGGGCAGCTGTGGCTTCTAGCTGGGCGGGTGGCATCCCTGTGACCCCTCCCCAGTGCCTCTCCTGGCCCTGGAAGTTGCCACTCCA
 V  E  G  S  C  G  F  **  G↑↑S
CACTCGCGTCGACACCGGAGAGGCCT↑AGGCGC
       88↓                  ↑CCT
```

9. BB9 (GHR SEQ) 18 5' GGAGGTCATAGACGTTGC 3'

43. BB43 (GH SEQ corrected BB8) 5' TCCAACCTAGAGCTGCTC 3'

87. BB87 (GH IGGFUS forward) 5> CGC AAG CTT GCC ACC ATG GCT
ACA GGC TCC CGG ACG >3

88. BB88 (GH IGGFUS rev) 5> CGC GGA TCC TCC GGA GAA GCC ACA
GCT GCC CTC CAC >3

EPO SEQ + 016605   OCTOBER 8, 1998

```
                         113   his                    89
              GGATCC ATG GGG GTG CAC GAA TGT CCT GCC TGG CTG TGG CTT CTC CTG TCC CTG CTG
              CCTAGG TAC CCC CAC GTG CTT ACA GGA CGG ACC GAC ACC GAA GAG GAC AGG GAC GAC
                     M   G   V   H   E   C   P   A   W   L   W   L   L   L  S  L   L


    TCG CTC CCT CTG GGC CTC CCA GTC CTG GGC GCC CCA CCA CGC CTC ATC TGT GAC AGC CGA GTC CTG
    AGC GAG GGA GAC CCG GAG GGT CAG GAC CCG CGG GGT GGT GCG GAG TAG ACA CTG TCG GCT GAG GAC
     S   L   P   L   G   L   P   V   L   G   A   P   P   R   L   I   C   D   S   R   V   L


    GAG AGG TAC CTC TTG GAG GCC AAG GAG GCC GAG AAT ATC ACG ACG GGC TGT GCT GAA CAC TGC AGC
    CTC TCC ATG GAG AAC CTC CGG TTC CTC CGG CTC TTA TAG TGC TGC CCG ACA CGA CTT GTG ACG TCG
     E   R   Y   L   L   E   A   K   E   A   E   N   I   T   T   G   C   A   E   H   C   S


    TTG AAT GAG AAT ATC ACT GTC CCA GAC ACC AAA GTT AAT TTC TAT GCC TGG AAG AGG ATG GAG GTC
    AAC TTA CTC TTA TAG TGA CAG GGT CTG TGG TTT CAA TTA AAG ATA CGG ACC TTC TCC TAC CTC CAG
     L   N   E   N   I   T   V   P   D   T   K   V   N   F   Y   A   W   K   R   M   E   V


                  54
    GGG CAG CAG GCC GTA GAA GTC TGG CAG GGC CTG GCC CTG CTG TCG GAA GCT GTC CTG GGG GGC CAG
    CCC GTC GTC CGG CAT CTT CAG ACC GTC CCG GAC CGG GAC GAC AGC CTT CGA CAG GAC GCC CCG GTC
     G   Q   Q   A   V   E   V   W   Q   G   L   A   L   L   S   E   A   V   L   R   G   Q


    GCC CTG TTG GTC AAC TCT TCC CAG CCG TGG GAG CCC CTG CAG CTG CAT GTG GAT AAA GCC GTC AGT
    CGG GAC AAC CAG TTG AGA AGG GTC GGC ACC CTC GGG GAC GTC GAC GTA CAC CTA TTT CGG CAG TCA
     A   L   L   V   N   S   S   Q   P   W   E   P   L   Q   L   H   V   D   K   A   V   S
                                                                       55


    GGC CTT CGC AGC CTC ACC ACT CTG CTT CGG GCT CTG GGA GCC CAG AAG GAA GCC ATC TCC CCT CCA
    CCG GAA GCG TCG GAG TGG TGA GAC GAA GCC CGA GAC CCT CGG GTC TTC CTT CGG TAG AGG GGA GGT
     G   L   R   S   L   T   T   L   L   R   A   L   G   A   Q   K   E   A   I   S   P   P


    GAT GCG GCC TCA GCT GCT CCA CTC CGA ACA ATC ACT GCT GAC ACT TTC CGC AAA CTC TTC CGA GTC
    CTA CGC CGG AGT CGA CGA GGT GAG GCT TGT TAG TGA CGA CTG TGA AAG GCG TTT GAG AAG GCT CAG
     D   A   A   S   A   A   P   L   R   T   I   T   A   D   T   F   R   K   L   F   R   V


    TAC TCC AAT TTC CTC CGG GGA AAG CTG AAG CTG TAC ACA GGG GAG GCC TGC AGG ACA GGG GAC AGA
    ATG AGG TTA AAG GAG GCC CCT TTC GAC TTC GAC ATG TGT CCC CTC CGG ACG TCC TGT CCC CTG TCT
     Y   S   N   F   L   R   G   K   L   K   L   Y   T   G   E   A   C   R   T   G   D   R
                                                               90


    TGA CCAGGTGTCCACCTGAGCATAACTACCAGAATTC
    ACT GGTCCACAGGTGGACTCGTATTGATGGTCTTAAG
```

54. BB54 (epo sq forward) 5> CAGCAGGCCGTAGAAGTC >3

55. BB55 (epo sq reverse) 5> ACTGACGGCTTTATCCAC >3

89. BB89 (EPO IGGFUS forward) 5> CGC AAG CTT GCC ACC ATG GGG
    GTG CAC GAA TGT CCT >3

90. BB90 (EPO IGGFUS reverse) 5> CGC GGA TCC TCC GGA TCT GTC
    CCC TGT GCA GGC >3

Witnessed & Understood by me,   Date   Invented by   Date
                                       10/19/98   10/19/98
                                                       Recorded by

111

OCTOBER 8, 1998

PCR's for EPO & GH CLONING FOR IgG FUSION

GH: **EPO**

GH		EPO	
20 µl	10x PCR buffer (PBO)	20 µl	10x PCR buffer PBO
20 µl	10x dNTP's (2mm)	20 µl	10x dNTPs
12 µl	25 mm MgCl₂	12 µl	25 mm MgCl₂
109.5 µl	H₂O	139.5 µl	H₂O
4 µl	BB 89 (40 pmol) p168	4 µl	BB 89 (40 pmol) p168
4 µl	BB 88 (") "	4 µl	BB 90 (") "
1 µl	Taq Pol (5u) (PRO)	1 µl	Taq Pol (5u) (PRO)
0.5 µl	Pfu Pol (1.25 u) (STRAT)	0.5 µl	Pfu Pol (1.25u) (STRAT)
1 µl	cDNA Hu Pituitary (1ng)	1 µl	pBB373 (1ng)
	(CLONTECH)		(pUC19 :: EPO (cDNA)

SPLIT INTO 3 x 66 µl   SPLIT INTO 3 x 66 µl

CYCLE:

96°C	3 MIN		SPDOM - 1:35
95°C	1 MIN		DOWN 3:30
(56, 61, 66)°C	1½ MIN	30x	
72°C	1 MIN		
6°C	HOLD		

RUN OUT: 1.2% Ag 5k 1x TBE 80V SPDOM 3:35
                                                  STOP-4:35

LANE

1	λ/4 + ØX			BOTTA
2	GH (84/88) (56°)	9/66 µl		HOOK
3	" " (61°)	"		(BOOM)
4	" " (66°)	"		
5				STORE
6	EPO (84/90) (56°)	6/66 µl		(RXN'S
7	" " (61°)	"		ON @ -20°C
8	" " (66°)	"		

Witnessed & Understood by me,	Date	Invented by	Date
Joe L	10/14/98	Recorded by	10/14/98

From Page No. 170

OCTOBER 9, 1998

USE 66°C RXNS (6H + EPO) FOR CLONING-;

Clean up WITH Qiagen PCR PURIF ; Elute 90u

Digest vs Bam HI + Hind III   (VECTOR)

~85ul each PCR   Digest pCDNA3 (G+)
                                    FOR cloning-
  10 ul   10X NEB2
  1 ul   100X BSA   15ul   pCDNA3 (G+) @
  2 ul   BamHI (yellow) 73ul   H₂0
  2ul   Hind III (clear) 10ul   10X NEB2
                          1 ul   BSA (2
@ 37°C ~9:25   1ul   H...

        ↓   @ 37°C ~9:25
      11:55   → 0.25

                          ADD 1ul EIP (10u) N
E+OH PREIP -   let go @ 37°C
4ml H₂0 + 1 ul EB w
70% 270u 870u   11:25 - PULL DIG
                          CLEAN UP WITH
AIR DRY 37°C ~20 mR   Qiagen PCR PURIF + 1

NaₐS₃0 20ul 1X w3   Elute 60ul EB +
                                 15 ul Do w3
+ MIN dup
    prep w2   + MIN out propgel

OCTOBER 9, 1998

PREP GELS (GH, EPO, GCSF) PCR FRAGS FOR

FUSION TO JoG4's + pcDNA3.1(+) VECTOR

0.7% A, SK 1X TAE   100 V   START - 12:15
                                      STOP - 1:30

LANE

1 pcDNA3.1(+) BamH+HindIII + R1P   1
2   "   "   "
3   "   "   "
4
5 pcDNA3.1(+) NOT cut
6   λ / H
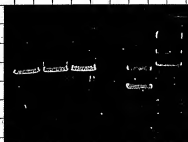
                     STOP ?
                     ACTUALLY

1.2% A, SK 1X TAE   100 V   START 12:55
                                 STOP   1:55

LANE
1 EPO PCR 89/90 (66°) X BamH HindIII   1
2
3 GH PCR 89/55 (66°) X BamH HindIII
4
5 GCSF PCR 91/92 (66°) X BamH HindIII
6   ΦX

EXCISE 1. Elute in QIAGEN II

pcDNA 2 SLICES ?, Elute 25µl X 2
                   Pool 20µl x 2, Prep

PCR3 — 1 SLICE EACH, Elute 25µl ; pull 20µl

        GO TO L-graph

OCTOBER 9, 1998

Ligations: GH, EP, CS PCRs FOR IG T145/WR

lygs 10/9 — (3µl PCDNA (HindIII+BamHI+ExI) gp (172)
2µl 5X BRL lg buff
2.5µl H₂O
0.5µl T4 ligase BRC

+ 2µl H₂O = lig coN
+ 2µl PCR 87/88 × (HindIII+Bam), gp ≡ lig GH
+ 2µl " 89/90 × (") gp ≡ " EP
+ 2 " 91/92 × (") gp ≡ " CS

@ 15°C ~4:30p 10/9

10/11 Tfm lygs 10/9

4µl each lig, 4µl ERB (BL21) + 36µl comp DH5α
T:0 ADD cells to DNA ON ICE
≡10 min, heat shock 42°, 45 sec, challenge
dil 1/10 w SOC inc @37°C ~60 min rd
PLATE: lygs 10µl, 100µl LB Ap¹⁰⁰
BLNK 100µl " "
@ 37°C ON ~ 8:30p

10/12 PULL plates looks good
STORE ON @ 4°C

EXHIBIT D

Project No._____
Book No._____
TITLE SDS Page of Protein A Col '12199 transfer

From Page No. B-1690 SDS PAGE   2/9/99

Gel 1 (NUMBERED)

Lane	Sample
1	Protein Non stnd std
2	MT
3	Load
4	31-
5	FW 2
6	3
7	4
8	5
9	6
10	7
11	8
12	9/10

Gel 2 (RED)

Lane	Sample
1	BioRad std
2	MT
3	Load
4	31-
5	FW 2
6	3
7	4
8	5
9	6
10	7
11	8
12	9/10

Sample Prep:

in 40 fac 20 µl + 5 µl SYSDTT-5% 2ME   load all
2 frac 10 µl load + 5 µl SYSB4-5% 2ME   "

heat 100° 5 min

Witnessed & Understood by me,	Date	Invented by	Date
Mary S. ___	8/11/98	___	2/10/99
		Recorded by	

TITLE 2/2/99 Transfec. Purification

Project No. _____
Book No. _____

From Page No. ____ pBBST 17a (EPO Jgb1(CH1)   2/16/99

Pool CM from Days 3,6,9 & 12
~ vol = 269 ml
Conc. on YM 30 membrane c Amicon Stirred Cell. 4°C 55 psi

1/ vol =
Wash _____ ml 20mM NaPi pH 7.0
Final vol = _____ ml

2 Load auto 1ml HiTRAP rProtein A Col.
   Flr= 0.5ml/min   Collect flt as 1 frac.

   Wash c 10ml 20mM NaPi pH 7.0   Collect as 1 frac
        1ml/min
   Elute @ 1ml/min c 0.1 M NaCitrate pH 3.0. Collect 0.5 ml
          frac. into 0.1ml 1M Tris pH 9.0 to neutralize

   √ pH of frac. & Adj. c 1M Tris pH 9.0 as needed to get pH to ~ 7.0

   √ on gel.

To Page No. 184

Witnessed & Understood by me,   Date   Invented by   Date
May 5 12   8/17/99   _____   2/16/99
                                            Recorded by

134

Project No. _____
Book No. _____   TITLE 2/2/99 Transfer Purific. SDS PH Protein A/01

om Page No. 83

prota_M1_UV_280nm_01 — prota_M2_Cond_01 — Fractions_1

2/2/99 Transfer. pBHF 179 CM a 3,6,9612 Protein A Col 1 HiFP
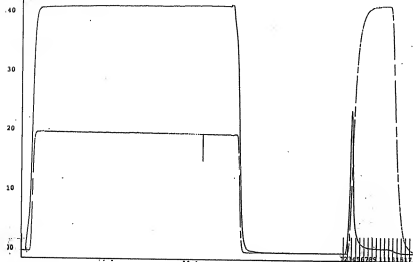
ser: DARIN   02-16-1999 04:08AM
ethod file: prota.frs

**** Run description *****
ample
2/2/99 Transfection pBHF 179 CM'a 3,6,9612

oop
50ml Superloop

column
1ml HiTrap rProtein A column

luent A
20mM NaPi pH 7.0

luent B
0.1M NaCitrate pH 3.0

radient

low rate
Load at 0.6ml/min Wash and elute at 1.0ml/min

raction size
Flo-thru as 1frac wash as 1frac elute as 0.5ml fractions

ther

ethod notes

un notes

valuation notes

ser: DARIN   02-16-1999 02:54AM
ethod file: effi.fmt

ethod base   : ml

**** Main method *****
 0.00 FLOW   0.6   {}
 0.00 CONC_B   0   {}
 0.00 INJ_VALVE   Load   {}
 0.10 INJ_VALVE   Inject   {}
28.00 FLOW   1.00   {}
28.00 INJ_VALVE   Load   {}
38.00 CONC_B   0   {}
38.00 CONC_B   100   {}
43.00 FLOW   1   {}
43.00 CONC_B   100   {}
43.00 CONC_B   0.0   {}
48.00 FLOW   0.02   {}

essed & Understood by me,	Date	Invented by		Date	To Page No.
	8/17/95			2/16/99	
		Recorded by			

TITLE SDS - PAGE Prot. A Col   Project No._____

Book No._____   185   1

From Page No.____ B-16% SDS-PAGE   2/18/99

GEL 1 (NonRed)

Lane	Sample
1	BioRad NonStainedStnd
2	mT
3	ProteinA Col Load
4	J/T
5	J/N 2
6	3
7	4
8	5
9	6
10	7
11	8
12	9/10

GEL 2 (RED)

Lane	Sample
1	BioRad Stnd
2	mT
3	Col Load
4	J/T
5	J/N 2
6	3
7	4
8	5
9	6
10	7
11	8
12	9/10

Sample Prep

Single Samples   20µl Sample + 5µl SDS BH - S/02µE   Load All

2) For Col   10µl   "   +   "   "   "   "   " "

heat 100°C 5 min

Witnessed & Understood by me,   Date   Invented by   Date

Mary S m   8/17/5   2/18/99

Recorded by

Project No._____
Book No._____ **TITLE** 1/4kB Transfer Purification

From Page No._____   pBRT 180 (CPO-IgG1(hinge))   2/17/99

Pool CM's from Day 3,6,9 & 12
Vol = 270ml
Conc on YM30 membrane Amicon Stirred Cell
  ↓ to 27ml
  Wash c 5ml 20mM NaPi pH7.0. Final Vol = 32ml ∴ 8.4x Conc

Run on 1ml Hi Trap r Protein A Col. Equib c 100mM NaCitrate pH3.0 + 20mM NaPi pH7.0
Load at 0.6ml/min Collect ↓T as 1 frac
Wash @ 1.0ml/min 10 Col Vol Collect as 1 frac
Elute c 0.1M NaCitrate pH3.0. @ 1ml/min. Collect 0.5ml frac into 0.1ml 1M TRIS
                    pH9.0 to Neutralize
↓ pH of frac & Adjust c 1M Tris pH9.0 to ≈ pH7.0

Witnessed & Understood by me,	Date	Invented by	Date	To Page No.____
Mary S. R	2/17/99	[signature]	2/18/99	
		Recorded by		

Method file: effl.fmt
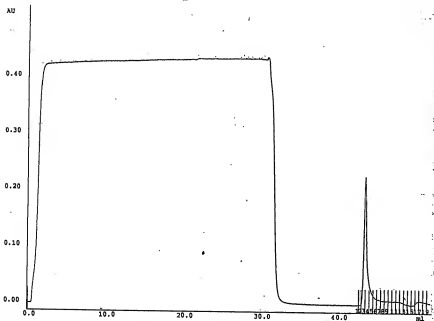
Method base : ml

***** Main method *****
```
 0.00 FLOW          0.5    ()
 0.00 CONC_B        0      ()
 0.00 INJ_VALVE     Load   ()
 0.10 INJ_VALVE     Inject ()
32.00 FLOW          1.00   ()
32.00 INJ_VALVE     Load   ()
42.00 CONC_B        0      ()
42.00 CONC_B        100    ()
47.00 FLOW          1      ()
47.00 CONC_B        100    ()
47.00 CONC_B        0.0    ()
52.00 FLOW          0.02   ()
```

Method file: prota.tre

***** Run description *****
Sample
 2/4/99 Transfection pBBT 180 CM's 3,6,9,12 pool

Loop
 50ml Superloop

Column
 1ml HiTrap rProtein A column

Eluent A
 20mM NaPi pH 7.0

Eluent B
 0.1M NaCitrate pH 3.0

Gradient

Flow rate
 Load at 0.6ml/min Wash and Elute at 1ml/min

Fraction size
 Flow thru as 1frac wasg as 1frac Elution as 0.5ml fraction

Other

Method notes
 Conc. CM pool from 2/4/99 Transfection run on 1ml Hitrap rProtein A column

Run notes

Evaluation notes

itnessed & Understood by me,	Date	Invented by	Date
Mauy S n	5/17/97	Recorded by	2/18/99

To Page No.___

Project No._____
Book No._____
TITLE SDS PAGE 2/4/99 Transfer Purif.

| From Page No. | B-1640 SDS PAGE | | | | | | | | | | 2/17/99 |

Gel 1 (unred)

Lane	Sample
1	Biorad Stnd
2	MT
3	Protein A Col Load
4	F/T
5	W 2
6	3
7	4
8	5
9	6
10	7
11	8
12	9/10

Gel 2 (red)

Lane	Sample
1	Biorad Stnd
2	MT
3	Protein A Col Load
4	F/T
5	W 2
6	3
7	4
8	5
9	6
10	7
11	8
12	9/10

Sample Prep:
Col Single Samples   20 µl + 5 µl SX SDS/0.2M ß   Load All
Sample pool   10 µl ea + 5 µl SX SB/1-S% 2 ME   Load All

heat 105°C 5 min

	Date	Invented by	Date
Witnessed & Understood by me,	8/17/9?		2/17/99
M___ ___		Recorded by	

To Page No._____

EXHIBIT E

From Page No. 95   FEBRUARY 26, 1999

SEQ DATA (NOW) 99-3   OK   BamHI-2SacII

SO SUBCLONE THIS FRAG. INTO EXPRESSION
VECTORS FOR   GH - IgG4 hinge
             GCSF - "   "   "
             EPO - "   "   "
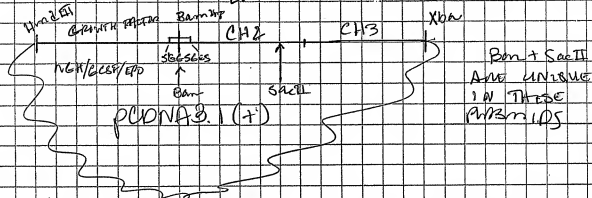
GH fus Ig G4 hinge = pBBT 163
GCSF "   "   "   " = pBBT 175
EPO "   "   "   "   = pBBT 181

99-3 = QSP 4 (2/22/99) now = pBBT 182

Digest 163, 175, 181 (1-2ug) vs Bam + SacII + CIP
                              + g-p VECTOR frag

     "   182 vs BamHI + SacII + gp - 525 bp frag

     Clone 182 525 bp into 163, 75, 181 VECTORs

Witnessed & Understood by me,   Date 3/5/99   Invented by   Date 3/3/99
                                                 Recorded by

EXHIBIT F

From Page No.___			pBBT	184	G-CSF Jγ4-CH	3/23/99
Setup Cos-1 Transfections			pBBT	185	EPO Jγ4-CH	

Use pBBT's from Endoprep 3/4/99

pBBT	Conc		for 700 μl of 0.1 μg/μl	TE
184	1.4 μg/μl		50 μl	650 μl
185	1.5 μg/μl		47 μl	653 μl

Setup 9/2 Mixes - for both pBBT 184+185

	/T-75	for 7 T-75's
DNA		
Plasmid DNA (0.1μg/μl)	93.5 μl	655 μl
Lipofectamine	70.1 μl	491 μl (Lot.No KM1Y07)
OptiMEM	2174 μl	15.22 ml

Invert + Swirl to mix   Let sit at RT for 45 min

*

1. Rinse flasks 1x c̄ Opti-Mem 10ml/flask
   replace c̄ 7.7ml Opti-Mem 1 flask

2. Add 2.3ml T/S Mix / flask = 10ml final vol
   37°C 4 hr   start @ 10:15.

3. After 4 hr   Aspirate off T/S Mix. Replace c̄ 10ml
   DMEM complete / flask
   => 37°C O/N

4. Cells look to be ~45-50 % confluent   (cells were plated on 3/22/99. 1¹
   flasks @ 2×10⁶ cells/flask. Used cells from 3/18/99 Pg.43)

Witnessed & Understood by me,	Date	Invented by	Date
Mary S R	9/2/9	Recorded by	3/23/99

E 3/23/99 Transfection

n Page No._____   3/24/99

Change Media on 3/23/99 transfection
1. Wash 2x c̄ 10ml wash DMEM Complete no additives
   replace c̄ 10ml DMEM P/S/Glut 1% Jlk.

Both sets of flasks look good. All are ~75-80% Confluent. Some areas of
flasks are more sparse (probably inoculator level issue)
                                                            DAY =

Change Media on 3/23/99 transfec's   (pBBT 184 + 185)   3/27/99
All Flask look OK   ~90% Confluent   CHO is yellow/orange
                                     Lots of floating cells.
#7 CM 5min 1000rpm - transfer to new falcon tubes. pull 500µl sample
   all => -20°C   vol ~ 70ml for each CM
                                                            DAY = 3

Change media on 3/23/99 Transfec's   (pBBT 184 + 185)   3/30/99
pBBT 184 - Flask #1 has mold contamination. Bleach Discard. Other flasks look
         OK. a few only went to ~ 80% confluent area 1
#7 CM 5min 1000rpm + Transfer to new falcon tubes. pull 500µl sample
   all => -20°C   vol ~ 70ml for Each pBBT #185   -20°C
                        +60ml for pBBT 184
                                                            DAY = 6

Change media on 3/23/99 Transfec.   (pBBT 184 + 185)   4/2/99
pBBT 184 - Flask #2 now has mold Contam. bleach + discard. Other
         flasks are still OK.
                                                            DAY = 9

Harvest Day 12 CM: 4/5/99
pBBT 184 - cells look OK. Shade & rounded cells present. CM is yellow.
           no contamination.
       185 - cells look OK   same as pBBT 184

#7 5min 1000rpm. pull Supe   500µl => -80°C
                             remain => -20°C

Discard Cells

Witnessed & Understood by me,	Date	Invented by	Date
Yays R	9/6/98	Dan SF	4/5/99
		Recorded by	

m Page No.____   Purify   EPO.IgG4·CH (pBBT 185)   4/22/99

Thaw CH from ³/23/99 transfec Days 3,6,9&12
VO1 = 270 ml
Conc on PM 3 membrane on Amicon Stirred cell

↓ to 31 ml   wash c̄ 6 ml filtrate
final vol = 37 ml   = 7.3 x conc.

run on 1 ml rProtein A col HiTRAP.   Equilib c̄
1. 20 mM NaPi pH 7.0
2. 0.1 M NaAcetate pH 3.0
3. 20 mM NaPi pH 7.0

prota_M1_UV_280nm_01 ---- Fractions_1 ----- Fraction_StartStop

Wash c̄
20 mM NaPi

Elute c̄ 0.1 r
NaAcetate
pH 3.0
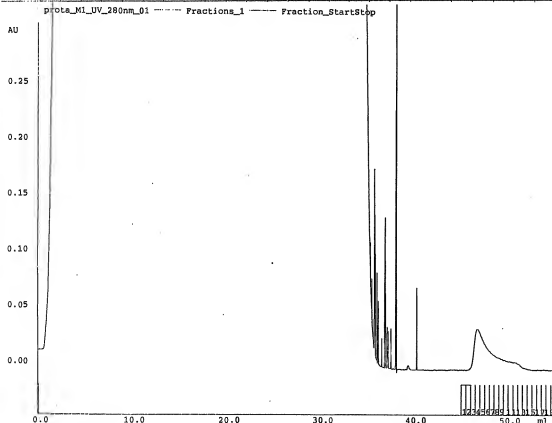
```
**** Main method *****
 0.00  FLOW         0.6
 0.00  CONC_B       0
 0.00  INJ_VALVE    Load
 0.10  INJ_VALVE    Inject
35.00  FLOW         1.00
35.00  INJ_VALVE    Load
45.00  CONC_B       0
45.00  CONC_B       100
50.00  CONC_B       1
50.00  CONC_B       100
50.00  CONC_B       0.0
55.00  FLOW         0.02
```

Load at 0.6 ml/min
Wash + elute at 1 ml/min

Vinc's   Flow Thru   1 frac
↓   Wash   1 frac
          Elution   0.5 ml frac

Collect elution frac
w/10 10 µl 8×DHTru
pH 9.0 To Page No. 62

Jays S.   9/2/99   4/29/99

Recorded by

Project No._____
Book No._____ TITLE 3/28/99 Transfer Purf. Gels.

4/28/99

Adjusted pH of frac to ~7.5 c̅ either 1.0M TrispH9.0 or 0.1M NaAcetate pH4.0

Run Samples on 8-16%) SDS-PAGE   Gel 1 NR   Gel 2 Red

Lane	Sample
1	mw Stnd
2	Col loaded
3	FT
4	3 2 3
5	4
6	5
7	6
8	7
9	8
10	9
11	10
12	11/12
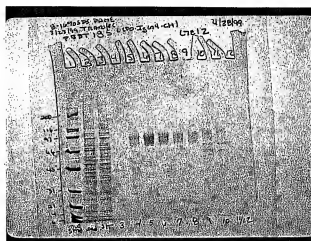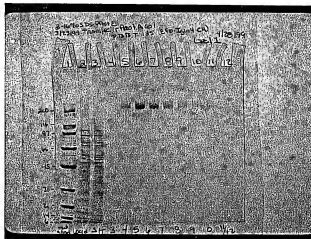
Sample Prep:
Load FT & Single frac   20µl + 5µl 5×SB/H-5%2ME
      2frac pool   10µl + 5µl 5×SB/H-5%2ME

heat All at 100°C 5min

To Page No._____

Witnessed & Understood by me,	Date	Invented by	Date
May S R	9/2/99	Recorded by	4/30/99

EXHIBIT G

Department of Health and Human Services
Public Health Service

Small Business Innovation Research Program

Phase II Grant Application

Follow Instructions carefully.

Leave blank — for PHS use only.		
Type	Activity	Number
Review Group		Formerly
Council Board *(Month, year)*		Date Received

1a. TITLE OF APPLICATION *(Do not exceed 56 typewriter spaces)*
Growth Factor Conjugates For Treating Hormonal Diseases

1b. Phase I Grant No.
1R43 DK54561-C

2. PRINCIPAL INVESTIGATOR
☐ New Investigator

2a. NAME *(Last, first, middle)*
Cox, George Norbert

2b. DEGREE(S)
B.A. Ph.D.

2c. SOCIAL SECURITY NO.
Provide on Personal Data Page

2d. POSITION TITLE
Chief Scientific Officer

2e. MAILING ADDRESS *(Street, city, state, zip-code)*
Bolder Biotechnology, Inc.
678 West Willow Street
Louisville, CO 80027

2f. TELEPHONE AND FAX *(Area code, number, and extension)*
TEL: 303-735-2296
FAX: 303-492-8731

BITNET/INTERNET Address:
BolderBio@aol.com

3. HUMAN SUBJECTS
☒ NO
☐ YES

3a. If "Yes," Exemption no. __ or __
IRB approval date __

3b. Assurance of compliance no. __

☐ Full IRB or
☐ Expedited Review

4. VERTEBRATE ANIMALS
☐ NO
☒ YES

4a. If "Yes," IACUC approval date
pending

4b. Animal welfare assurance no.
none

8. PERFORMANCE SITES *(Organizations and addresses)*

Bolder Biotechnology, Inc.
Porter Biosciences Building, Suite A140
University of Colorado
Boulder, CO 80309

9. APPLICANT ORGANIZATION *(Name and address of applicant small business concern)*

Bolder Biotechnology, Inc.
678 West Willow Street
Louisville, CO 80027

10. ENTITY IDENTIFICATION NUMBER
84-1372278

Congressional District
Colorado, 2nd

12. SMALL BUSINESS CERTIFICATION
☒ Small Business Concern ☐ Women-owned
☐ Socially and Economically Disadvantaged

11. INVENTIONS AND PATENTS ☐ Previously reported OR
☒ NO ☐ YES If "Yes," ☐ Not previously reported

13. NOTICE OF PROPRIETARY INFORMATION: The information identified by asterisks(*) on pages 5-7, 14-17, 20-47 of this application constitutes trade secrets or information that is commercial or financial and confidential or privileged. It is furnished to the Government in confidence with the understanding that such information shall be used or disclosed only for evaluation of this application, provided that, if a grant is awarded as a result of or in connection with the submission of this application, the Government shall have the right to use or disclose the information herein to the extent provided by law. This restriction does not limit the Government's right to use the information if it is obtained without restriction from another source.

14. DISCLOSURE PERMISSION STATEMENT: If this application does not result in an award, is the Government permitted to disclose the title only of your proposed project, and the name, address, and telephone number of the official signing for the applicant organization, to organizations that may be interested in contacting you for further information or possible investment? ☒ YES ☐ NO

15. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
Name: George N. Cox
Title: President & CSO
Address:
Bolder Biotechnology, Inc.
678 West Willow Street
Louisville, CO 80027

Telephone: 303-735-2296
FAX: 303-492-8731
BITNET/INTERNET Address:
BolderBio@aol.com

16. PRINCIPAL INVESTIGATOR ASSURANCE: I certify that the statements herein are true, complete, and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

SIGNATURE OF PERSON NAMED IN 2a
(In ink. "Per" signature not acceptable.)
George N. Cox

DATE
4/14/99

17. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete, and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Service terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF PERSON NAMED IN 15
(In ink. "Per" signature not acceptable.)
George N. Cox

DATE
4/14/99

PHS 6246-2 (Rev. 1/98)

Face Page

*Research Plan:
*A. Specific aims:
*   There is considerable interest on the part of patients and healthcare providers in the development of low cost,
*long-acting, "user-friendly" protein therapeutics. Most protein pharmaceuticals have short circulating half-lives in
*the body and must be injected daily or every other day for maximum effectiveness. For example, Growth
*Hormone (GH) and granulocyte colony-stimulating factor (G-CSF) require daily injections and erythropoietin
* (EPO) requires every other day injections for maximum effectiveness. These recombinant proteins have proven
*extremely effective at treating short stature and cachexia (GH), neutropenia (G-CSF) and anemia (EPO). For each
*of these proteins it is known that increasing the circulating half-life of the protein improves the protein's *in vivo*
*performance. We propose to create longer-acting forms of GH, G-CSF and EPO through covalent fusion of these
*proteins to the heavy chain domain of human IgG1. Human IgG1 has a long serum half-life, on the order of 21
*days. Fusion of several other proteins, principally extracellular domains of cell surface receptors, to the IgG1 heavy
*chain domain has resulted in increased serum half-lives for these proteins. Despite this success, IgG fusion protein
*technology has been applied in only a few instances to cytokines and growth factors. Fully active IL-2- and IL-10-
*IgG fusion proteins have been constructed, but data are not available for other cytokines and growth factors.
*   During Phase I, we created recombinant fusion proteins comprising GH, G-CSF and EPO fused to the Fc
* (Hinge-CH2-CH3) and C_H (complete heavy chain: CH1-Hinge- CH2- CH3) domains of human IgG1 and IgG4.
*The fusion proteins were expressed as secreted as dimeric proteins from transiently transfected mammalian cells.
*The fusion proteins were purified and their bioactivities compared to the corresponding non-fusion proteins in
*appropriate *in vitro* mammalian cell proliferation assays. On a molar basis, the EPO-IgG-Fc and G-CSF-IgG-Fc
*fusion proteins had biological activities essentially identical to EPO and G-CSF in the *in vitro* bioassays. In
*contrast, bioactivities of the GH-IgG-Fc fusion proteins were reduced 6-10-fold compared to GH. Biological
*activities of all of the IgG-C_H fusion proteins were reduced approximately 3-fold relative to the activities of the
*corresponding IgG-Fc fusion proteins. The lower activities of the IgG-C_H fusions appeared to be due to
*aggregation of the proteins during purification. The Phase I studies have identified the EPO-IgG-Fc and G-CSF-
*IgG-Fc fusion proteins as excellent candidates for further development. During Phase II we will concentrate efforts
*on these proteins and do not propose further studies of the GH-IgG fusion proteins at this time.
*   During the Phase II portion of the grant, initial work will focus on characterizing the *in vivo* properties of the
*EPO-IgG1-Fc and G-CSF-IgG1-Fc fusion proteins. We will immediately manufacture sufficient quantities of these
*fusion proteins for *in vivo* studies. We will measure the pharmacokinetic properties of the fusion proteins and
*compare their relative effectiveness to wild type EPO and G-CSF in normal and diseased animals. The animal
*studies will determine the effects of different dosing regimens on the *in vivo* effectiveness of the proteins. Our goal
*is to create fusion proteins that are equal or superior to the natural proteins in stimulating biological activities *in
vivo, but which require less frequent dosing, on the order of once every two to four weeks, rather than daily or
*every other day. Previous studies suggest it should be possible to achieve this goal by fusion of the proteins to the
*heavy chain domain of human IgG1.
*   A second aspect of the Phase II studies will be to optimize specific features of the fully active EPO-IgG-Fc and
*G-CSF-IgG-Fc fusion proteins.   In particular we will eliminate or minimize the linker between the growth factor
*and the IgG domain. If the animal studies indicate that complement and Fc receptor binding properties of the IgG
*domain causes toxicities *in vivo* we will introduce mutations that eliminate or reduce these functions.
*   A third aspect of the Phase II studies will be to develop high level expression systems for manufacture of the
*fusion proteins using stably transformed mammalian cells. We will optimize procedures for purifying the proteins
*and develop analytical methods for characterizing their purity and structural properties.
*   A fourth aspect of the Phase II portion of the grant will be to continue efforts to construct fully active EPO-
*IgG1-C_H and G-CSF-IgG-C_H fusion proteins, since these proteins should have even longer circulating half-lives
*than the corresponding IgG-Fc fusions. We believe we understand why the IgG1-C_H fusion proteins are
*aggregating and propose experiments to rectify the problem and create fully active versions of these proteins. If
*fully active EPO- and G-CSF-IgG1-C_H fusion proteins are constructed, we will characterize their *in vivo* properties.
*   The primary goal of the Phase II portion of the grant is to identify the best EPO-IgG and G-CSF fusion proteins
*for commercial development. The specific tasks proposed for Phase II are:
*   1. Prepare several hundred micrograms of each of the EPO-IgG1-Fc and G-CSF-IgG1-Fc fusion proteins
*for pharmacokinetic and animal efficacy studies.
*   2. Prepare several hundred micrograms each of the control, non-fused EPO and G-CSFproteins for
*pharmacokinetic and animal efficacy studies.
*   3. Perform pharmacokinetic experiments to demonstrate increased circulating half-lives of the fusion
*proteins relative to the non-fused proteins.

*   4. Compare the relative effectiveness of EPO-IgG-Fc and EPO in stimulating red blood cell formation in
*normal and anemic animals.
*   5. Compare the relative effectiveness of G-CSF-IgG-Fc and G-CSF in stimulating neutrophil formation in
*normal and neutropenic animals.
*   6. Create stably-transfected mammalian cell lines expressing the most effective EPO-, G-CSF- and GH-IgG
*fusion proteins.
*   7. Determine whether it is possible to eliminate or minimize the linker sequence between the growth factor
*and the IgG domain without reducing biological activities of the fusion proteins.
*   8. Continue efforts to create fully active EPO- and G-CSF- IgGI-C_H fusion proteins.
*   9. Perform pharmacokinetic and animal efficacy experiments with any new fully active EPO- or G-CSF-
*IgG-C_H fusion proteins (i.e. repeat tasks 1-5 above).

*B. Significance
*   There is considerable interest on the part of patients and healthcare providers in the development of low cost,
*long-acting, "user-friendly" protein therapeutics. Proteins are expensive to manufacture and unlike conventional
*small molecule drugs, are not readily absorbed by the body. Therefore, proteins must be administered by injection.
*Most proteins are cleared rapidly from the body, necessitating frequent, often daily, injections for optimum
*effectiveness. This is the case for GH and G-CSF. Some proteins including EPO are effective when administered
*less often (three times per week for EPO) but this is due to the fact that the proteins are glycosylated. Patients
*dislike injections, which leads to reduced compliance and reduced drug efficacy. The length of time an injected
*protein remains in the body is determined by the protein's size and whether or not the protein contains covalent
*modifications such as glycosylation. Circulating concentrations of injected proteins change constantly, often by
*several orders of magnitude, over a 24 hour period. Rapidly changing concentrations of protein agonists can have
*dramatic downstream consequences, at times understimulating and at other times overstimulating target cells.
*Similar problems plague protein antagonists. These fluctuations can lead to decreased efficacy and increased
*frequency of adverse side-effects for protein therapeutics. The rapid clearance of recombinant proteins from the
*body significantly increases the amount of protein required per patient and dramatically increases the cost of
*treatment. The cost of human protein pharmaceuticals is expected to increase dramatically in the years ahead as
*new and existing drugs are approved for more disease indications. Current word-wide sales of protein therapeutics
*are in excess of $10 billion annually and are growing at a greater than 10% annual rate. Thus, there is a strong
*need to develop protein delivery technologies that lower the costs of protein therapeutics to patients and healthcare
*providers. One solution to this problem is the development of methods to prolong the circulating half-lives of
*protein therapeutics in the body so that the proteins do not have to be injected frequently. This solution also
*provides patients and healthcare providers with protein therapeutics that are "user-friendly", i.e., that do not
*require frequent injections.

* EPO is a 35-39 kDa glycoprotein secreted by the adult kidney. The mature human protein contains 165 amino
*acids and is heavily glycosylated. EPO is the hormone primarily responsible for stimulating erythropoiesis or red
*blood cell formation. EPO acts on immature red blood cell precursors to stimulate their further proliferation and
*differentiation into mature red blood cells. Recombinant human EPO is used to restore red blood cell production in
*patients with anemia resulting from renal failure, chemotherapy and drug complications. EPO recently received
*FDA approval for stimulating red blood cell formation in patients undergoing certain types of elective surgeries.

*U.S. sales of EPO exceeded $2 billion and world-wide sales exceeded $3 billion in 1997. The protein is
*administered by thrice weekly intravenous (dialysis patients) or subcutaneous (non-dialysis patients) injections.
*   The structure of porcine GH has been solved by X-ray crystallography (Abdel-Meguid et al., 1987). The
*protein has a compact globular structure, comprising four amphipathic alpha helical bundles joined by loops and
*human GH has a similar structure (de Vos et al., 1992). The three dimensional structure of G-CSF has been
*determined by X-ray crystallography (Hill et al., 1993; Lovejoy et al., 1993) and resembles that of GH, comprising
*four amphipathic alpha helical bundles joined by loops. The extensive glycosylation of EPO has precluded
*crystallographic studies but Cheetham et al. (1998) recently reported NMR based structural studies and described a
*four helix bundle structure very similar to those of GH and G-CSF.
*   GH has two receptor binding sites and binds two receptor molecules (Cunningham et al., 1991; de Vos et al.,
*1992). Dimerization of GH receptors is required for activation of the intracellular signaling pathways that lead to
*cellular responses to GH. It is believed that G-CSF (Fukunaga et al., 1991) and EPO (Matthew's et al., 1996)
*dimerize their receptors in a manner similar to the way GH dimerizes its receptor.
*   Boissel et al (1993) showed that fusion of a six amino acid poly-histidine tag to the C-terminus of EPO did not
*interfere with EPO bioactivity which suggested that other C-terminal fusions, such as those proposed in this grant
*application, would be active. The principal investigator is unaware of data addressing the question of whether C-
*terminal fusions of GH or G-CSF are active.

* Phase I: Determine whether IgG fusion proteins of GH, EPO and G-CSF are biologically active
*   We proposed to create IgG fusion proteins of GH, EPO and G-CSF with extended half-lives, increased efficacy
*and improved safety. To achieve this goal we needed to determine whether IgG fusions of these proteins were
*biologically active and if so, how their bioactivities compared to bioactivities of the non-fused proteins. Previous
*studies suggested that the C-terminus was not important for EPO bioactivity, but data for GH and G-CSF were not
*available. During Phase I we constructed fusions of GH, EPO and G-CSF with the C_H and Fc domains of IgG1.
*We also constructed fusions of these growth factors with the corresponding domains of human IgG4. The goal of
*the Phase I studies was to identify at least one IgG fusion protein of GH, EPO or G-CSF that retained complete or
*near complete (within two-fold of wild type) *in vitro* biological activity.

*Relationship of Phase I to Phase II Studies
*   As described below, the Phase I studies have allowed us to determine that certain IgG fusion proteins of EPO
*and G-CSF retain complete *in vitro* bioactivity. These results have demonstrated the feasibility of creating growth
*factor-IgG fusion proteins that retain high activity. In contrast, the GH-IgG fusion proteins displayed significantly
*reduced *in vitro* biological activity compared to GH. Therefore, during Phase II we will focus efforts on the fully
*active EPO- and G-CSF- IgG fusion proteins and not pursue additional experiments with the GH-IgG fusion
*protein. The Phase II studies are designed to demonstrate the superior performance of the EPO-IgG and G-CSF-
*IgG fusion proteins relative to the non-fused proteins in animal disease models. Positive data from the Phase II
*animal experiments will provide the proof-of-concept needed for us to negotiate a partnering agreement with a
*large pharmaceutical/biotechnology company to rapidly bring long-acting, highly potent EPO-IgG and G-CSF-IgG
*fusion proteins to market.

3. Phase I Final Report

*Project funding period: October 1, 1998 – May 31, 1999
*Personnel: The personnel who contributed to this work are listed below.

Person	Role	Hours Devoted to Project
George Cox, Ph.D.	Principal Investigator	578 hr
Daniel Doherty, Ph.D.	Scientist	403 hr
Darin Smith	Research Associate	570 hr

[a] Through March 31, 1999

*Summary of Accomplishments
*   The goal of the Phase I portion of the grant was to identify one or more fully active IgG fusions (Fc or C_H
*fusion) of GH, EPO or G-CSF. The specific tasks involved were:

*1. Clone cDNAs encoding GH, G-CSF and EPO and fuse DNAs encoding these proteins to DNAs encoding the Fc
*   or complete heavy chain (C_H) region of human IgG1.
*2. Clone DNAs encoding the fusion proteins into a mammalian cell expression vector.
*3. Transiently transfect mammalian cells with DNAs encoding the fusion proteins and purify the secreted fusion
*   proteins to homogeneity using Protein A affinity chromatography, followed by other chromatographic
*   procedures, if needed.
*4. Characterize the fusion proteins by polyacrylamide gel electrophoresis under reducing and non-reducing
*   conditions to determine whether the fusion proteins are dimeric.
*5. Measure bioactivities ($EC_{50}s$) of the fusion proteins using appropriate GH-, G-CSF-, and EPO- responsive
*   mammalian cell lines in culture. Compare bioactivities of the fusion proteins and non-fused control
*   proteins in the bioassays.

*   All of these objectives have been met and the primary goal has been exceeded. The six IgG1- Fc and IgG1-C_H
*fusions have been constructed, expressed in mammalian cells and the recombinant fusion proteins purified and
*analyzed. In addition, we also constructed fusions of GH, EPO and G-CSF to the Fc and C_H domains of IgG4,
*which has certain properties, e.g., reduced affinity for complement and Fc receptors relative to IgG1, which may be
*desirable for fusion protein therapeutics. The EPO and G-CSF IgG1-Fc and IgG4-Fc fusion proteins were found to
*be as active as EPO and G-CSF in appropriate mammalian cell bioassays. These fusion proteins were excellent
*candidates for further consideration. The EPO- and G-CSF-IgG1-C_H fusion proteins were found to have reduced
*biological activities (2- to3-fold) relative to non-fused EPO and G-CSF. Reduced bioactivities of these proteins
*appeared to be due to aggregation of the fusion proteins during purification. All of the GH-IgG fusion proteins had
*4 -17-fold reduced biological activities relative to unfused GH, suggesting that the IgG domain may interfere with
*the binding of GH to its receptor. A detailed summary of these experiments is provided below.

*I. Construction of GF-IgG gene fusions.
*I. A. Strategy. Twelve growth factor (GF)–IgG gene fusions were constructed. The general strategy employed for
*these constructions is outlined here and the specifics of individual cloning steps are detailed below. Cloning of the
*IgG4-C_H coding sequence involved additional variations to the general strategy and these variations are described
*below. The human growth factor genes (GH, EPO and GCSF) were cloned as cDNAs from various RNA sources
*detailed below. PCR primers used in these clonings added an optimized Kozak sequence (GCCACCC) and a Hind
*III restriction site to the 5' end of each these clones and a portion of a flexible peptide linker (ser-gly-gly-ser)
*terminating in a Bam HI restriction site, to the 3' end of each of these clones. The growth factor genes were cloned
*as Hind III – Bam HI fragments into the mammalian cell expression vector pCDNA3.1(+) (Invitrogen, Inc.) and
*sequenced. In parallel, IgG coding sequences (IgG1-Fc, IgG1-C_H, IgG4-Fc, IgG4-C_H) were cloned from cDNAs
*generated from human leukocyte RNA. PCR forward primers used in these clonings incorporated a portion of a
*flexible peptide linker (gly-ser-gly-gly-ser) containing a Bam HI restriction site at the 5' end of each of these
*clones. The reverse PCR primers were designed to anneal to the 3' untranslated regions of the IgG1 and IgG4
*mRNAs (about 40 bp downstream of the translational stop codon) and included an Xba I restriction site. The IgG
*coding sequences were cloned into pCDNA3.1(+) as Bam HI – Xba I fragments and confirmed by DNA
*sequencing. The fusion genes were then constructed by excising the IgG coding sequences as Bam HI – Xba I
*fragments and cloning these fragments into the pCDNA::GF recombinant plasmids that had been cut with Bam HI
*and Xba I. In the resulting pCDNA3.1 constructs the fusion genes are transcribed by the strong cytomeglovirus

*immediate early promoter present in pCDNA3.1(+) upstream of the cloned fusion gene. Ligation of the two
*fragments through the *Bam* HI site within the linker sequence results in a seven amino acid linker (ser-gly-gly-ser-
*gly-gly-ser) at the fusion junction.

pCDNA3.1	growth factor	linker	IgG	pCDNA3.1
Hind III		*Bam* HI		*Xba* I

*I. B. Cloning of growth factor cDNAs.
*   Cloning of hGH: A cDNA encoding hGH was amplified from human pituitary single-stranded cDNA
*(CLONTECH, Inc.), using the polymerase chain reaction (PCR) technique and primers BB87 (5>
*CGCAAGCTTGCCACCATGGCTACAGGCTCCCGGACG >3) and BB88 (5> CGCGGATCCTCCGGAGAA
*GCCACAGCTGCCCTCCAC >3). Primer BB87 anneals to the 5' end of the coding sequence for the hGH
*secretion signal, whereas the reverse primer, BB88, anneals to the 3' end of the GH coding sequence. The
*resulting ~ 680 bp PCR product was digested with *Hind* III and *Bam* HI, gel purified and cloned into
*pCDNA3.1(+) vector that had been digested with *Hind* III and *Bam* HI, alkaline phosphatase treated, and gel
*purified. A clone with the correct DNA sequence was designated pCDNA3.1(+)::GHfus or pBBT159.
*   Cloning of EPO. We previously cloned in pUCl9 a cDNA encoding human EPO from the human liver
*cell line Hep3B. The DNA sequence of the EPO gene was confirmed and the plasmid designated pBBT131. This
*plasmid was used as template in a PCR reaction with primers BB89 (5>CGCAAGCTTGCCACCATGGGGGTGC
*ACGAATGTCCT >3) and BB90 (5>CGCGGATCCTCCGGATCTGTCCCCTGTCCTGCAGGC >3) to construct
*a modified EPO cDNA suitable for fusion with IgG genes. Primer BB89 anneals to the 5' end of the coding
*sequence for the EPO secretion signal and the reverse primer, BB90, anneals to the 3' end of the EPO coding
*sequence. The resulting ~ 610 bp PCR product was digested with *Hind* III and *Bam* HI, gel purified and cloned
*into pCDNA3.1(+) vector that had been digested with *Hind* III and *Bam* HI, alkaline phosphatase treated, and gel
*purified. A clone with the correct DNA sequence was designated pCDNA3.1(+)::EPOfus or pBBT176.
*   Cloning of G-CSF. A cDNA encoding G-CSF was amplified by PCR from total RNA isolated from the
*human bladder carcinoma cell line 5637 (American Type Culture Collection). The cells were grown in Roswell
*Park Memorial Institute (RPMI) 1640 media supplemented with 10% FBS, 50 units/ml penicillin and 50 µg/ml
*streptomycin. RNA was isolated from the cells using an RNeasy Mini RNA isolation kit purchased from Qiagen,
*Inc. (Santa Clarita, CA) following the manufacturer's directions. First strand synthesis of single-stranded cDNA
*was accomplished using a 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) from Boehringer Mannheim Corp
*and random hexamers were used as the primer. Subsequent PCR reactions using the products of the first strand
*synthesis as template were carried out with forward primer BB91 (5>CGCAAGCTTGCCACCATGGCTGGACC
*TGCCACCCAG>3 and reverse primer BB92 (5>CGCGGATCCTCCGGAGGGCTGGGCAAGGTGGCGTAG
*>3). Primer BB91 anneals to the 5' end of the coding sequence for the G-CSF secretion signal and the reverse
*primer, BB92, anneals to the 3' end of the G-CSF coding sequence. The resulting ~ 640 bp PCR product was
*digested with *Hind* III and *Bam* HI, gel purified and cloned into pCDNA3.1(+) vector that had been digested with
Hind III and *Bam* HI, alkaline phosphatase treated, and gel purified. A clone with the correct DNA sequence was
*designated pCDNA3.1(+)::G-CSFfus or pBBT165.

*I. C. Cloning of IgG coding sequences.
*   Cloning of IgG1-Fc coding sequences. A cDNA encoding IgG1-Fc (hinge-CH2-CH3 domains) was
*amplified from human leukocyte single-stranded cDNA (CLONTECH) by PCR using primers BB83
*(5>CGCGGATCCG GTGGCTCAGAGCCCAAATCTTGTGACAAAACT >3) and BB82 (5>CGCTCTAG
*AGGTACGTGCCAAGCA TCCTCG>3). Forward primer BB83 anneals to the 5' end of the coding sequence of
*the hinge domain of IgG1, whereas the reverse primer BB82 anneals to the 3' untranslated region of IgG1 and
*IgG4 mRNA ~ 45 bp downstream of the translational stop codon. The IgG1 and IgG4 sequences are identical over
*the 21 bp segment to which BB82 anneals. The ~ 790 bp PCR product was digested with *Bam* HI and *Xba* I, gel
*purified and cloned into pCDNA3.1(+) vector that had been digested with *Bam* HI and *Xba* I, alkaline phosphatase
*treated, and gel purified. Two clones were sequenced but each contained a single base pair substitution that
*resulted in an amino acid substitution mutation. Otherwise the sequences matched the published human IgG1
*genomic DNA sequence (Ellison et al., 1982). The relative positions of the mutations in the two clones allowed us
*to use convenient unique restriction sites (*Sac* II in the CH2 domain of IgG1 and *Bgl* II in the pCDNA3.1(+)

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vector) to construct a full length IgG1-Fc clone in pCDNA3.1(+) via *in vitro* recombination. The resulting clone, which had the correct IgG1-Fc sequence, was designated pCDNA3.1(+)::fusIgG1-Fc or pBBT167.

Cloning of IgG4-Fc coding sequences. A cDNA encoding IgG4-Fc (hinge-CH2-CH3 domains) was amplified from human leukocyte single-stranded cDNA (CLONTECH) by PCR using primers BB84 (5>CGCGGATCCGG TGGCTCAGAGTCCAAATATGGTCCCCCATGC>3) and BB82 (5>CGCTCTAG *AGGTACGTGCCAAGCA TCCTCG>3). Forward primer BB84 anneals to the 5' end of the coding sequence of the hinge domain of IgG4. The reverse primer BB82 is described above. The ~ 790 bp PCR product was digested with *Bam* HI and *Xba* 1 and cloned into pCDNA3.1(+) that had been similarly digested. A clone with the correct DNA sequence (Ellison et al., 1981) was designated pCDNA3.1(+)::fusIgG4-Fc or pBBT158.

Cloning of IgG1-C$_H$ coding sequences. A cDNA encoding IgG1-C$_H$ (CH1-hinge-CH2-CH3 domains) was amplified from human leukocyte single-stranded cDNA (CLONTECH) by PCR using BB81 (5>CGCGGATCC *GGTGGCTCAGCCTCCACCAAGGGCCCATC>3) and BB82 (5>CGCTCTAGAGGTACGTGCCAAGC *ATCCTCG>3). Forward primer BB81 anneals to the 5' end of the coding sequence of the CH1 domain of IgG1 and IgG4. The sequences at the 5' end of the CH1 domains of these two exons are almost identical: 19/20 nucleotides match. The reverse primer, BB82, is described above. The ~ 1080 bp PCR product was digested with *Bam* HI, gel purified and cloned into pCDNA3.1(+) that had been digested similarly. These primers in principle could amplify both IgG1 and IgG4 sequences. Since IgG1 is much more abundant in serum than IgG4 (Paul, 19xx) we expected that most clones would encode IgG1. The first two clones sequenced were IgG1 but each contained a single base pair substitution that resulted in an amino acid substitution mutation. Otherwise the sequences obtained matched the published human IgG1 genomic DNA sequence (Ellison et al., 1982). The relative positions of the mutations in the two clones allowed us to use convenient unique restriction sites (*Age* I in the CH1 domain of IgG1 and *Bst* BI in the pCDNA3.1(+) vector) to construct a full length IgG1-C$_H$ clone in pCDNA3.1(+) via *in vitro* recombination. A clone with the correct IgG1-C$_H$ sequence was designated pCDNA3.1(+)::fusIgG1-C$_H$ or pBBT166.

Cloning of IgG4-C$_H$ coding sequences. The near identity of the DNA sequences encoding the 5' ends of the IgG1 and IgG4 CH1 domains and the relatively low abundance of the IgG4 mRNA led us to an alternative strategy for cloning the IgG4-C$_H$ coding sequences. We used PCR-based site directed mutagenesis to change the DNA sequence of the cloned IgG1 CH1 domain to match the amino acid sequence of the IgG4 CH1 domain. The CH1 domains differ at only 8 of 98 nucleotides and these positions are clustered, so that one round of PCR using two mutagenic oligos can convert the IgG1 CH1 sequence into the IgG4 CH1 sequence. A second round of PCR added the *Bam* HI site and linker sequence to the 5' end of the IgG4 CH1 and 21 bp of sequence from the IgG4 Hinge domain to the 3' end. The technique of "gene splicing by overlap extension" (Horton et al., 1993) was then employed to recombine the engineered IgG4 CH1 domain with the IgG4 Fc (Hinge-CH2-CH3) sequence. In this technique two separate fragments sharing a segment of identical sequence, the "overlap", at one end are extended through the annealed overlap regions in a PCR reaction as diagrammed below.

To construct the IgG4 CH1 sequence, mutagenic primers BB119 (5> TCCACCAAG GGCCCATCCGT *CTTCCCCCTGGCGCCCTGCTCCAGGAGCACCTCCGAGAGC ACAGC>3) and BB120 (5> TCTCTTG *TCCACCTTGGTGTTGCTGGGCTTGTGATC TACGTTGCAGGTGTAGGTCTTCGTGCCCAA >3) were used in PCR reactions with pBBT166, which carries the cloned IgG1-C$_H$ sequence as described above. Forward primer BB119 anneals to the sequence encoding amino acids 2 through 23 of the CH1 domain and encodes 4 amino acid substitutions: S14C, K16R, G20E and G21S. Reverse primer BB120 anneals to the sequence encoding amino acids 76 through 97 of the CH1 domain and encodes 4 additional amino acid substitutions: Q79K, I82T, N86D and K97R. The ~290 bp product of this PCR reaction was gel purified and used template in a PCR reaction with 8primers BB81(see above) and BB121(5>TGGGGGACCATATTTG GACTCAACTCTCTTGTCCACCTT >3). Reverse primer BB121 anneals to the 3' end of the CH1 domain of IgG4, adds amino acid 98 of the CH1 domain and 21 bp extending into the Hinge domain of IgG4. The ~ 330 bp product of this reaction was gel purified and used as one of the template molecules in the PCR splicing reaction. The other template for the splicing reaction

*was generated by PCR of the cloned IgG4-Fc sequence of pBBT158 (described above) with primers BB84 and
*BB82 which amplify the IgG4 Fc domain as described above. The resulting ~ 790 bp product consists of the IgG4
*hinge-CH2-CH3 sequence. This fragment was gel purified and used as one of the template molecules in the PCR
*splicing reaction. This reaction employed the primers BB81 and BB82 and generated a full-length "spliced"
*product of ~ 1075 bp. To minimize the DNA sequencing required to confirm this product, the PCR fragment was
*digested with Bam HI and Sac II and the ~ 530 bp fragment (containing the complete CH1 and hinge domains and
*a portion of the CH2 domain) was cloned into pBC-SK+ (Stratagene) for sequencing. The sequence of the Bam HI
*– Sac II fragment was confirmed for one clone which was the designated pBBT182. The Bam HI – Sac II fragment
*of pBBT182 was then used convert the GF-IgG4-Fc clones to full length GF-IgG4-C$_H$ clones as detailed below.

*I. D. Construction of GF-IgG fusions. Most (9/12) of the growth factor-IgG gene fusions were generated by
*excising the IgG4 coding sequences cloned in pCDNA3.1(+) as Bam HI – Xba I fragments and cloning these
*fragments into the pCDNA::[GF] recombinant plasmids which had been cut with Bam HI and Xba I. The fusions
*of the three growth factor genes to IgG4-C$_H$ were constructed by excising the ~ 530 bp Bam HI–Sac II fragment of
*pBBT182 and replacing the ~ 240 bp Bam HI- Sac II fragments of the three pCDNA::[GF]-IgG4-Fc clones. The
*resulting plasmids and the GF-IgG fusion proteins they encode are listed in Table 1.

*II. Expression and Purification of GF-IgG Fusion Proteins
*II. A. Small Scale Transfection of COS Cells
*   Expression and bioactivity of the GF-IgG fusion proteins were assessed initially by small-scale transfection of COS
*cells. Endotoxin-free plasmid DNAs were prepared using a Qiagen "Endo-Free Plasmid Purification Kit" according to
*the vendor protocol and used to transfect COS-1 cells (ATCC). The COS-1 cells were Delbecco's Modified Eagle's
*Media supplemented with 10% FBS, 50units/ml penicillin, 50µg/ml streptomycin and 2mM glutamine (growth media).
*Initial transfection experiments were carried out in Costar 6 well tissue culture plates using the following protocol.
*Briefly, 2-3 x 10^5 cells were seeded into each well in 2 ml of growth media and allowed to incubate overnight at 37°C
*and 5% CO$_2$ by which time the cells had reached 50-60% confluency. For each well, 0.8 µg of plasmid DNA was
*complexed with 6 µl of LipofectAMINE reagent (Gibco BRL) in 186 µl of OPTI-MEM I Reduced Serum Medium
*(Gibco BRL) for 30-45 minutes at room temperature. COS-1 cells were washed one time with 2ml of OPTI-MEM I per
*well and then 1.8 ml of OPTI-MEM I was added to each well. The complex mixture was then added to the well and left
*at 37°C, 5% CO$_2$ for approximately 4-5 hours. After the incubation period, the mixture was replaced with 2 ml of
*growth media per well and left overnight at 37°C, 5% CO$_2$. The next day the cells were washed twice with 2ml of
*DMEM (no additives) per well. Following the wash steps, 2 ml of serum-free growth media was added to each well
*and the cells left at 37°C, 5% CO$_2$. Conditioned media containing the GF-IgG-fusion proteins were harvested after 72
*hours and analyzed by SDS-PAGE and Western blot to confirm expression of the GF-IgG-fusion proteins. The parent
*plasmid, pCDNA 3.1(+) (Invitrogen) was used as a negative control. Transfection efficiency was estimated to be
*~15%, using pCMVβ (Clontech), which expresses $E.$ $coli$ β-galactosidase. Transfected cells expressing
*β-galactosidase were identified using a β-Gal Staining Set (Boehringer Mannheim).
*   Samples of the conditioned media were prepared in SDS-PAGE sample buffer with the addition of 1% β-
*mercaptoethanol (BME) when desirable and electrophoresed on precast 14% Tris-glycine polyacrylamide gels
*(Novex). Western blots using appropriate antisera demonstrated expression of all of the GF-IgG fusion proteins
*(data not shown – see purified proteins below). The GH-IgG fusion proteins were detected using a polyclonal
*rabbit anti-synthetic-hGH antiserum (kindly provided by Dr. A.F. Parlow and the National Hormone and Pituitary
*Program). The EPO- and G-CSF-IgG fusion proteins were detected using polyclonal antisera purchased from
*R&D Systems. Serial dilutions of the conditioned media were analyzed in the appropriate in $vitro$ bioassays
*described later. These assays demonstrated significant activity in the conditioned media (data not shown) and
*encouraged us to perform large-scale transfections so that the proteins could be purified for specific activity
*measurements.

*II. B. Large Scale Transfection of COS-1 Cells
*   Large scale transfections were carried out using Corning 100 mm tissue culture dishes or Corning T-75 tissue
*culture flasks. For 100 mm dishes, 1.6 x 10^6 cells were plated in 10 ml of growth media per dish and incubated at
*37°C, 5% CO$_2$ overnight. For each 100 mm dish, 6.7 µg endotoxin-free plasmid DNA was complexed with 50 µl
*of LipofecAMINE reagent in 1.5 ml of OPTI-MEM I for 30-45minutes at room temperature. The COS-1 cells
*were washed one time with 10 ml OPTI-MEM per dish and then replaced with 6.6 ml of OPTI-MEM I. Following
*complex formation, 1.67 ml of the complex was added to each dish and left at 37°C, 5% CO$_2$ for 4-5 hours. After

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*the incubation period, the reaction mixture was replaced with 10 ml of serum containing growth media per dish and
*left at 37°C, 5% CO_2 overnight. The next day the cells were washed twice with 10 ml of DMEM (no additives) per
*dish. Following the wash steps, 10ml of serum-free growth media was added to each dish and incubated at 37°C,
*5% CO_2. Conditioned media were harvested routinely every three days (on days 3, 6, 9 and 12) and fresh serum-
*free growth media added to the cells. Transfections in T-75 culture flasks were identical to the 100mm dish
*protocol with the following exceptions: Cells were plated at 2×10^6 cells per flask and 9.35 μg of endotoxin-free
*plasmid DNA was complexed with 70 μl of LipofectAMINE reagent in 2.1ml of OPTI-MEM I for each T-75 flask.
*Following complex formation, 2.3 ml of the complex was added to each flask containing 7.7 ml of OPTI-MEM I.
*Transfection efficiency was determined to be ~15% using pCMVβ and staining for β-galactosidase expression as
*described earlier. The 12 plasmids listed in Table 1 were transfected into COS-1 cells using the large-scale format
*to generate protein for purification. The conditioned media were clarified by centrifugation and stored at -20°C for
*later purification. Western blots were used to confirm expression of the IgG-fusion proteins.

*II. C. Purification of GF-IgG-Fusion Proteins
*   Approximately 300 ml of transfected COS-1 cell conditioned media for each IgG-fusion protein was pooled and
*concentrated using an Ultrafiltration cell and either a YM3 or YM30 DIAFLO Ultrafiltration membrane (Amicon).
*Concentrated pools were then loaded onto a 1ml Pharmacia HiTrap recombinant Protein A column previously
*equilibrated with 20 mM NaPhosphate pH 7.0. The column was washed with 20 mM NaPhosphate until the A_{280}
*had reached baseline. Bound protein was eluted with 100 mM NaCitrate pH 3.0 and collected into sufficient 1M
*Tris pH 9.0 to achieve a final pH of approximately 7.0. Each fusion protein was purified using a dedicated column
*to avoid any possibility of cross-contamination. All of the IgG fusion proteins chromatographed similarly, yielding
*a single peak in the elution step. Column fractions were analyzed using 8-16% precast Tris-glycine SDS-PAGE
*and fractions enriched for the IgG-fusion protein were pooled. A typical chromatogram and corresponding gel
*from the EPO-IgG1-Fc purification is shown in Figures 1A and 1B. Protein concentrations of the pooled fractions
*were determined by Bradford assay using bovine serum albumin (BSA) as the standard. Recoveries of the various
*purified GF-IgG fusion proteins are given in Table 1 and ranged from 96 to 376 μg per 300 ml of conditioned
*media.
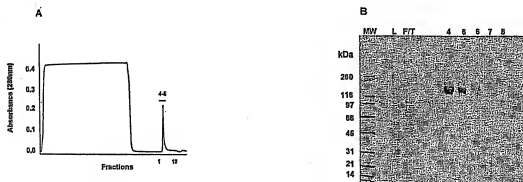
*Figure 1. rProtein A purification of EPO-IgG-Fc. Panel A shows the rProtein A column chromatogram. Panel B is non-reducing SDS-
*PAGE analysis of the rProtein A column fractions. MW (molecular weight standards), L (column load), F/T(column flow through), and
*column fractions 4-8.

*   To date, 9 of 12 GF-IgG fusion proteins have been purified to near homogeneity. Figure 2 displays SDS gels of
*the purified proteins under reducing and non-reducing conditions and stained with Coomassie blue. All of the
*GF-IgG fusion proteins were recovered principally as disulfide-linked dimers. The molecular weights of the
*proteins ranged from 115-190 kDa kDa under non-reducing conditions and 50-70 kDa under reducing conditions,
*largely consistent with the molecular weights predicted in Table 1. The molecular weights of the EPO-IgG fusion
*proteins were the only ones larger than predicted (see Figure 2), presumably due to extensive glycosylation of the
*EPO domain. Monomeric fusion proteins were more abundant with the IgG4-Fc fusion proteins (they can be seen
*in the non-reduced gels in Figure 2), but still represented the minority (less than 10%) of the protein in these
*preparations. The sizes of the major IgG fusion protein bands were different from the molecular weights of bovine

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*IgG (see Figure 2), indicating that the proteins purified were not contaminating bovine IgGs from serum used in the
*experiments. The major IgG fusion protein bands also reacted with antisera specific for GH, EPO and G-CSF in
*Western blots of the samples (data not shown). Purity of the IgG fusion proteins was estimated to be at least 90%
*from Coomassie blue staining of the gels.
*   All of the GF-IgG-CH fusions contained a large aggregate that migrated at the top of the gel when the samples
*were analyzed under non-reducing conditions. This aggregate disappeared when the samples were analyzed under
*reducing conditions and the amount of protein at the molecular weight of the major GF-IgG-C$_H$ bands seemed to
*increase proportionately. The aggregates also reacted with antisera specific for the various growth factors. These
*data suggest the aggregates are disulfide-linked multimers of the GF-IgG-C$_H$ fusion proteins. Under reducing
*SDS-PAGE conditions, all of the GF-IgG-C$_H$ fusions show a diffuse band approximately 20 kDa larger than the
*main GF-IgG-C$_H$ band. This band reacted with antisera against the growth factors and may be related to the
*aggregates.

*Figure 2. Analysis of Purified IgG-fusion proteins by SDS-PAGE. Panel A shows purified GH-IgG fusion proteins. Lane 1, molecular
*weight standards; Lanes 2 & 3 are bovine IgG standard at 1 & 2µg respectively, reduced; Lanes 4,5 & 6 are GH-IgG1-C$_H$, GH-IgG1-Fc, and
*GH-IgG4-Fc respectively, reduced; Lane 8, 2µg bovine IgG standard non-reduced; Lanes 9,10 & 11, are identical to lanes 4,5 & 6 except non-
*reduced. Panel B shows purified G-CSF-IgG fusion proteins. Lanes 1,2,3 & 8 are identical to those in Panel A; Lanes 4,5 & 6 are G-CSF-
*IgG1-C$_H$, G-CSF-IgG1-Fc and G-CSF-IgG4-Fc respectively, reduced; Lanes 9,10 & 11 are identical to lanes 4,5 & 6 except non-reduced.
*Panel C shows purified EPO-IgG fusion proteins. Lanes 1,2,3 & 8 are identical to those in Panel A; Lanes 4,5 & 6 are EPO-IgG1-C$_H$, EPO-
*IgG1-Fc and EPO-IgG4-Fc respectively, reduced; Lanes 9,10 & 11 are identical to lanes 4,5 & 6 except non-reduced.

*Table 1   Predicted Molecular Weights and Recoveries of GF-IgG Fusion Proteins

Expression Plasmid	IgG Fusion Protein	Predicted Molecular Weight (kDa) Monomer	Dimer	Protein Recovery (µg /500ml)
pBBT 171	GH-IgG1-C$_H$	58,706	117,412	376
pBBT 172	GH-IgG1-Fc	48,693	97,386	248
pBBT183	GH-IgG4-C$_H$	58,541	117,082	In Progress
pBBT 163	GH-IgG4-Fc	48,365	96,730	96
pBBT 173	G-CSF-IgG1-C$_H$	55,564	111,128	122
pBBT 174	G-CSF-IgG1-Fc	45,551	91,102	122
pBBT 184	G-CSF-IgG4-C$_H$	55,399	110,798	In Progress
pBBT 175	G-CSF-IgG4-Fc	45,222	90,444	96
pBBT 179	EPO-IgG1-C$_H$	54,972	109,944	133
pBBT 180	EPO-IgG1-Fc	44,960	89,920	235
pBBT 185	EPO-IgG4-C$_H$	54,808	109,616	In Progress
pBBT 181	EPO-IgG4-Fc	44,632	89,264	257

*¹ Does not include molecular weight contributions due to of glycosylation.

*III. Bioactivities of Purified IgG Fusion Proteins

*   Cell proliferation assays were developed to measure bioactivities of the IgG fusion proteins. The assays measure
*uptake and bioreduction of the tetrazolium salt MTS [3-(4,5-dimethylthiazol-2-yl)-5-3-carboxyphenyl)-2-(4-
*sulphenyl)-2H-tetrazolium]. In the presence of an electron coupler such as phenazine methosulfate (PMS), MTS is
*converted to a formazan product that is soluble in tissue culture media and can be measured directly at 490 nm.
*Cell number is linear with absorbance values up to about 2 (data not shown). The advantage of using MTS in the
*assays, rather than the more conventional tetrazolium salt MTT (Mosmann, 1983) is that absorbance of the wells
*can be determined without the need to lyse the cells with organic solvents, as is required for assays utilizing MTT.
*For EPO and G-CSF we were able to use existing cell lines to develop the bioassays. For GH, we needed to create
*a cell line that proliferates in response to GH. Such a cell line was created by stably transforming a murine
*leukemia cell line with a GH receptor.
*   In general, the bioassays were set up by washing the appropriate cells three times with media (no additives) and
*resuspending the cells at a concentration of 1×10^5/ml in media with additives (media used for each cell line is given
*below). Fifty µl (5×10^3 cells) of the cell suspension was aliquotted per test well of a flat bottom 96 well tissue
*culture plate. Serial dilutions of the protein samples to be tested were prepared in serum containing media. Fifty µl
*of the diluted protein samples were added to the test wells and the plates incubated at 37^0C in a humidified 5% CO_2
*tissue culture incubator. Protein samples were assayed in triplicate wells. After 60-72 h, 20 µl of an MTS/PMS
*mixture (CellTiter 96 AQueous One Solution, Promega) was added to each well and the plates incubate at 37^0C in
*the tissue culture incubator for 1-4 h. Absorbance of the wells was read at 490 nm using a microplate reader.
*Control wells contained media but no cells. Mean absorbance values for the triplicate control wells were
*subtracted from mean values obtained for the test wells. EC$_{50}$s, the amount of protein required for half maximal
*stimulation, was calculated for each sample and used to compare bioactivities of the proteins. Experiments were
*repeated at least three times for each protein. Non-glycosylated molecular weights were used in the molar ratio
*calculations for consistency; using molecular weights of the fusions estimated from SDS gels (50-70 kDa) and
*35kDa for EPO gave similar activity ratios. Non-glycosylated molecular weights of 18,936, 18,987 and 22,129
*were assumed for EPO, G-CSF and GH, respectively. Monomer molecular weights were used in the calculations
*for the IgG fusion proteins.

*III. A. EPO-IgG Fusion Proteins
* The human UT7/epo cell line was obtained from Dr. F. Bunn of Harvard Medical School, Boston, MA. This cell
*line proliferates in response to EPO and is dependent upon EPO for cell survival (Boissel et al., 1993). The cells
*were maintained in Iscove's Modified Delbecco's Media (IMDM) supplemented with 10% FBS, 50 units/ml
*penicillin, 50 µg/ml streptomycin and 1 unit/ml recombinant human EPO (CHO cell-expressed; R&D Systems).
*Bioassays were performed in cell maintenance media using the procedures described above. Serial dilutions of
*recombinant CHO cell-expressed human rEPO (R&D Systems) were analyzed in parallel.
* The UT7/epo cell line shows a strong proliferative response to rEPO, as evidenced by a dose-dependent increase
*in cell number and absorbance values (Figure 3). In the absence of rEPO, the majority of UT7/epo cells die, giving
*absorbance values less than 0.1. Commercial CHO cell-expressed rEPO had a mean EC$_{50}$ of approximately 0.6
*ng/ml in the bioassay (Table 2). This value agrees with EC$_{50}$ values reported in the R&D Systems specifications
*(0.05 – 0.1 unit/ml or approximately 0.4-0.8 ng/ml). The EPO-IgG1-Fc and IgG4-Fc fusion proteins had identical
*EC50's of approximately 1.3 ng/ml in the bioassay (Table 2). On a molar basis, the EC$_{50}$s of CHO-cell expressed
*rEPO and the EPO-IgG-Fc fusions were identical (approximately 30 pM; Table 2). The EPO-IgG1-C$_H$ fusion
*protein had an EC$_{50}$ of 3.1 ng/ml or 60 pM (Table 2), which represents an approximate 2-fold reduction in specific
*activity relative to the EPO-IgG-Fc fusion proteins and non-fused rEPO. Dose response curves for CHO cell-
*expressed rEPO and the EPO-IgG fusion proteins performed on the same day are shown in Figure 3.

*Table 2. Bioactivities of EPO-IgG Fusion Proteins

Clone	Protein	EC$_{50}$ Range[1] (ng/ml)	Mean EC$_{50}$[1] (ng/ml)	Mean EC$_{50}$[1] (pM)
-	rhEPO (CHO)	0.52, 0.55, 0.60	0.56	30
pBBT180	EPO-IgG1-Fc	1.1, 1.2, 1.5	1.27	28
pBBT181	EPO-IgG4-Fc	1.1, 1.2, 1.5	1.27	29
pBBT179	EPO-IgG1-C$_H$	2.9, 3.0, 3.5	3.13	57
pBBT185	EPO-IgG4-C$_H$	In Progress	-	-

[1] Data from individual experiments

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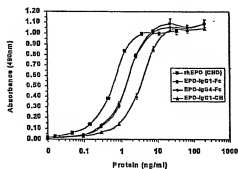
*Figure 3. Dose response curves for EPO-IgG fusion
*proteins and rEPO for stimulating proliferation of
*UT7/epo *cells. Data represent means +/- SD for
*triplicate wells.
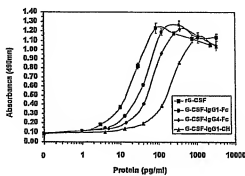
*Figure 4. Dose response curves for G-CSF-IgG
*fusion proteins and rG-CSF for stimulating
*proliferation of NFS60 cells. Data represent
*means +/- SD for triplicate wells.

IV. Summary of Phase I Experiments

The Phase I studies have revealed that it is possible to create IgG-Fc fusions of EPO and G-CSF with complete biological activity. IgG1-Fc fusions of both proteins had comparable biological activities to non-fused EPO and G-CSF. As expected, the IgG-Fc fusion proteins were predominantly dimeric, presumably joined through disulfide bonds in the hinge regions of the IgG domains. The fact that these fusion proteins were fully active suggests that both halves of the dimeric proteins are biologically active. The EPO-IgG4-Fc fusion protein also retained complete biological activity, but the G-CSF-IgG4-Fc fusion protein appeared to have slightly reduced biological activity.

The EPO-IgG1-C_H and G-CSF-IgG1-C_H fusion proteins also were potent growth factors *in vitro*, although their specific activities were reduced 2-3-fold relative to the non-fused proteins. These proteins also were predominantly dimeric. The reduced bioactivities of these proteins correlated with the presence of high molecular weight, disulfide-linked aggregates detected by SDS-PAGE analysis. Since these aggregates were not detected in the original conditioned media, we believe they formed during purification of the proteins. The aggregates were observed only with the IgG-C_H constructs and all of the IgG1-C_H constructs displayed aggregates. The IgG-C_H fusion proteins may be aggregating via the hydrophobic domain in the CH1 domain that normally packs against the IgG light chain (Traunecker et al., 1989). Aggregation appears to be accompanied by disulfide bond formation, possibly through the free cysteine in the CH2 domain that normally disulfide bonds with a cysteine in the IgG light chain. We propose experiments for Phase II to test this hypothesis and potentially improve/restore bioactivties of the IgG1-C_H fusion proteins to wild type.

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*V. Phase II Experimental Design and Methods
*   The Phase II experiments have several goals. The primary goal will be to demonstrate the superior performance
*of the EPO-IgG and G-CSF-IgG fusion proteins relative to non-fused EPO and G-CSF in in vivo models.
*Specifically, we propose to demonstrate increased circulating half-lives for the EPO- and G-CSF-IgG fusion
*proteins and efficacy equal to, or superior to, that of non-fused EPO and G-CSF, even when the fusion proteins are
*administered less frequently and in lower doses.   We believe these results are key to our moving forward to the
*next stage of commercial development of this technology in that it will allow us to negotiate a
*licensing/development agreement with a pharmaceutical or biotechnology company capable of rapidly bringing
*EPO-IgG and G-CSG-IgG fusion protein products to market. The initial In vivo experiments will test the EPO-
*IgG1-Fc and G-CSF1-IgG1-Fc fusion proteins since these proteins can be produced in adequate amounts and retain
*complete biological activity.
*   A second aspect of the proposed research will be to examine the need for a flexible linker between the growth
*factor domain and the IgG domain. We will attempt to eliminate or minimize this linker in an effort to create an
* "all human" molecule. If fully active fusion proteins lacking a linker or with a minimal linker are identified, we
*will perform additional In vivo experiments with best of these proteins.
*A third aspect of the Phase II research is to continue efforts begun during Phase I to construct fully active IgG-C_H
*fusion proteins.   We propose experiments to co-express IgG light chains to prevent aggregation of the fusion
*proteins. If these experiments are successful, we will perform additional animal studies with the these proteins.
*   A fourth aspect of the proposed research will be to develop processes for high level recombinant expression of
*the best fusion protein development candidates in stably transformed mammalian cells and to develop processes for
*purification and characterization of these recombinant proteins.

*I. Animal Experiments with IgG-Fc Fusion Proteins
*A. Pharmacokinetic Experiments
*   We will collaborate with researchers at BolderPATH, Inc., a local contract pharmacology, toxicology and
*pathology company, to perform pharmacokinetic studies of the EPO-IgG1-Fc and G-CSF-IgG1-Fc fusion proteins
*to determine if the fusion proteins have longer circulating half-lives than the corresponding non-fused proteins.
*BolderPATH, Inc., an unaffiliated company, is based at the University of Colorado, Boulder. The animal
*experiments will be performed at The University of Colorado, Boulder Animal Facility, which is an accredited
*animal research facility. The pharmacokinetic data will guide us in designing animal experiments to determine
*dosing regimens to compare the efficiencies of EPO-IgG1-Fc and G-CSF-IgG1-Fc to wild-type EPO and G-CSF.
*Both intravenous and subcutaneous pharmacokinetic data will be obtained. Terminal pharmacokinetic parameters
*will be calculated from the intravenous delivery data. These experiments will be performed initially with the IgG1-
*Fc fusion proteins and later with other promising development candidates such as modified versions of these
*proteins or fully active IgG-C_H constructs, if we are able to create them. We expect to find that these IgG fusion
*have significantly longer circulating half-lives than the non-fused proteins.
*   For the intravenous delivery studies, rats (~350g) will receive an intravenous bolus injection (0.1 mg/kg) of the
*IgG1-Fc fusion protein (EPO or G-CSF) or the corresponding non-fused protein (EPO or G-CSF) and circulating
*levels of the proteins measured over the course of 144 h. Three rats will be used for each protein sample. Blood
*samples will be drawn at 0, 0.08, 0.5, 1.5, 4, 8, 12, 24, 48, 72, 96, 120, and 144 h following intravenous
*administration. The large number of blood samples to be analyzed is necessitated by the fact that IgG fusion
*proteins can have half-lives of several days in a rat (Richter et al., 1999). The control proteins, EPO and G-CSF,
*are expected to have circulating half-lives of 2-3 h (Elliot and Byrne, 1995; Tanaka et al., 1991). Serum levels of
*the test proteins will be quantitated by Bolder Biotechnology researchers using commercially available EPO and G-
*CSF ELISA kits (R & D Systems). Because of the cost of the ELISAs, serial dilutions of each blood sample will be
*analyzed initially in the in vitro bioassays to identify dilutions that will fall within the linear range of the ELISAs.

*(0.025 to 1.6 ng/ml for EPO and 0.04 to 2.5 ng/ml for G-CSF). We will perform initial titration experiments to
*determine the relative sensitivity of the ELISA for detecting the IgG1-Fc proteins and the corresponding
*non-fused proteins. This experiment will require 105 μg of each protein.
*   The subcutaneous delivery studies will follow the same protocol as the intravenous studies except for the route
*of delivery. Serum levels of the test proteins will be quantitated by ELISA as described above. This experiment
*will require 105 μg of each protein.

*B. Animal Efficacy Models
*   Initially, we will determine *in vivo* efficacy of EPO-IgG1-Fc and G-CSF-IgG1-Fc in normal rats and mice since
*these models are easier to perform. These studies will allow us to determine proper doses and dosing schedules.
*Subsequently, we will determine efficacy of the proteins in appropriate disease models – anemia for EPO-IgG1-Fc
*and neutropenia for G-CSF-IgG1-Fc. We will collaborate with researchers at BolderPATH, Inc. to perform the
*animal studies, which will be performed at the University of Colorado, Boulder Animal Facility. BolderPATH has
*experience in performing these bioassays. We expect to find that the IgG fusion proteins produce results equal or
*superior to the non-fused proteins, but can achieve these results with less frequent dosing. We expect to find that
*the IgG fusion proteins are more efficacious than the non-fused proteins when both are administered using the less
*frequent dosing schedules.
*   The pharmacokinetic experiments will provide guidance in deciding dosing schedules for the IgG1 fusion
*proteins to be used for the animal studies. From published results with other IgG-Fc fusion proteins (Richter et al.,
*1999; Zeng et al., 1995) we expect the fusion protein will be effective when administered every other day or every
*third day and possibly less often, e.g. a single injection. Because rodents metabolize proteins faster than humans
* (Mordenti et al., 1991), dosing every other day or every third day in a rodent is roughly equivalent to dosing every
*week or every other week in a human. The dosing schedules may have to be modified depending upon the results
*of the pharmacokinetic studies and initial animal efficacy results. The dose of protein administered per injection to
*the rodents also may have to be modified based upon the results of the pharmacokinetic experiments and initial
*animal efficacy results. We have budgeted monies for experiments to determine whether the fusion proteins are
*effective when administered every other day (EOD) and every third day (ETD).

*1.   EPO Animal Efficacy Models
*   We will compare the *in vivo* efficacy of EPO-IgG1-Fc fusion proteins (and later other EPO-IgG fusion proteins)
*to wild type EPO in stimulating increases increases in hematocrit and erythropoiesis in normal rats. Sprague-
*Dawley rats (~200g) will be purchased from a commercial supplier such as Charles River (Wilmington, MA).
*Previous studies have shown that administration of 100 IU/kg (approximately 800 ng/kg) of rEPO once per day
* (160 ng SID / 200 g rat) by subcutaneous injection gives a significant increase in hematocrit and erythropoiesis in
*rodents (Matsumoto et al., 1990; Vaziri et al., 1994; Baldwin et al., 1998; Sykowski et al., 1998). Groups of 5 rats
*will receive subcutaneous injections of rEPO, EPO-IgG1-Fc or placebo (vehicle solution) at specified intervals for
*up to five days. The highest dose of EPO-IgG1-Fc will be a molar equivalent rEPO. We will test a wide range of
*EPO-IgG1-Fc doses (625-fold variation) for these initial experiments to increase the likelihood that one of the
*doses will be effective. It is possible that administration of too much EPO-IgG1-Fc will impede erythropoiesis due
*to toxicity. Control rats will receive vehicle solution only. Additional control groups will receive rEPO (160
*ng/SID for 5 days) and 160 ng rEPO using the same dosing regimen as EPO-IgG1-Fc. On day 6 the animals will be
*sacrificed and blood samples collected for hematocrit and complete blood cell count (CBC) analysis.
*Hematopoietic tissues (liver and spleen) will be collected, weighed and fixed in formalin for histopathologic
*analyses to look for evidence of increased erythropoiesis. Bone marrow will be removed from various long bones
*and the sternum for unit particle preps and histopathologic analysis to look for evidence of increased
*erythropoiesis. Comparisons between groups will be made using a Students T test for single comparisons and one-
*way analysis of variance for multiple comparisons. P< 0.05 will be considered significant.
*   We expect to find that daily injections of rEPO stimulate increases in hematocrit and erythropoiesis in the rats,
*whereas less frequent administration of the same dose of rEPO does not, or does so to a lesser extent. We expect to
*observe dose-dependent increases in these parameters in the EPO-IgG-Fc-treated animals. We expect to observe
*greater increases in these parameters in the EPO-IgG1-Fc-treated animals than in animals treated with EPO using
*the less frequent dosing schedules. We also expect to find that significantly less EPO-IgG1-Fc is required to
*achieve the same increases in these parameters obtained with daily injections of EPO.
*   We will consider performing additional experiments with less frequent dosing, e.g., a single injection, if results
*from the EOD nad ETD experiments are promising.

*EPO Experiment 1 – Normal Rats – Every Other Day Dosing: Rats will receive injections every other day
*(EOD), i.e. on days 1, 3 and 5, for a total of three injections. Total amount of rEPO and EPO-IgG1-Fc required for
*Experiment 1 is 6.4 µg and 8 µg, respectively.

Group	Sample (Dose and Frequency)	Number of Rats	Protein Required
1	Vehicle solution (EOD)	5	0
2	EPO (160 ng SID)	5	4.0 µg
3	EPO (160 ng EOD)	5	2.4 µg
4	EPO-IgG1-Fc (0.64 ng EOD)	5	0.0096 µg
5	EPO-IgG1-Fc (3.2 ng EOD)	5	0.048 µg
6	EPO-IgG1-Fc (16 ng EOD)	5	0.24 µg
7	EPO-IgG1-Fc (80 ng EOD)	5	1.2 µg
8	EPO-IgG1-Fc (400 ng EOD)	5	6.0 µg

*EPO Experiment 2 – Normal Rats – Every Third Day Dosing: Rats will receive injections every third day
*(ETD), i.e., on days 1 and 4, for a total of two injections. Total amount of rEPO and EPO-IgG1-Fc required for
*Experiment 2 is 5.6 µg and 5 µg, respectively.

Group	Sample (Dose and Frequency)	Number of Rats	Protein Required
1	Vehicle solution (ETD)	5	0
2	EPO (160 ng SID)	5	4.0 µg
3	EPO (160 ng ETD)	5	1.6 µg
4	EPO-IgG1-Fc (0.64 ng ETD)	5	0.0064µg
5	EPO-IgG1-Fc (3.2 ng ETD)	5	0.032 µg
6	EPO-IgG1-Fc (16 ng ETD)	5	0.16 µg
7	EPO-IgG1-Fc (80 ng ETD)	5	0.8 µg
8	EPO-IgG1-Fc (400 ng ETD)	5	4.0 µg

*EPO Experiment 3 - Rat Anemia Model
*   Cisplatin-induced anemia is a well-characterized rodent model of chemotherapy-induced anemia and has direct
*relevance to the human clinical setting. rEPO reverses the anemia in this model when administered at daily doses
*of 100 Units/kg (Matsumoto et al., 1990; Vaziri et al., 1994; Baldwin et al., 1998). If EPO-IgG-Fc is capable of
*increasing hematocrit levels in normal rats we expect it also will be effective at reversing anemia in this model.
*The dosing schedule for EPO-IgG-Fc to be used in this experiment will be the one that worked best in the normal
*rat experiments.   The experimental protocol outlined below assumes EPO-IgG-Fc will be effective when
*administered ETD, but this can be altered based upon results of experiments with normal rats. Sprague-Dawley
*rats (~200g) will be treated on day 0 with an intraperitoneal injection of Cisplatin (3.5mg/kg) to induce anemia and
*randomized to various treatment groups. The dosing schedule and amounts of protein injected per rat will be as
*described for the ETD normal rat experiments described above. Rats will receive injections of EPO-IgG-Fc, rEPO
*or saline on days 1, 4 and 7, for a total of three injections. One control group will receive daily
*subcutaneous injections of rEPO (100 Units/kg). Another control group will not receive the initial Cisplatin
*injection but will receive ETD injections of saline. On day 9 the rats will be sacrificed and blood and tissue
*samples obtained for comprehensive CBC and histopathology analyses. The amount of rEPO and EPO-IgG-Fc
*required for this experiment is 9 µg and 7.5 µg, respectively.

Group	Cisplatin Treatment	Sample (Dose and Frequency)	Number of Rats	Protein Required
1	-	Vehicle solution (ETD)	5	-
2	+	Vehicle solution (ETD)	5	-
3	+	EPO (160 ng SID)	5	6.4 µg
4	+	EPO (160 ng ETD)	5	2.4 µg
5	+	EPO-IgG1-Fc (0.64 ng ETD)	5	0.0096µg
6	+	EPO-IgG1-Fc (3.2 ng ETD)	5	0.048 µg
7	+	EPO-IgG1-Fc (16 ng ETD)	5	0.24 µg
8	+	EPO-IgG1-Fc (80 ng ETD)	5	1.2 µg
9	+	EPO-IgG1-Fc (400 ng ETD)	5	6.0 µg

*C. Prepare Recombinant Proteins for Pharmacokinetic and Animal Efficacy Experiments

*1. EPO-IgG1-Fc and G-CSF-IgG1-Fc Fusion Proteins

*   The total amounts of the IgG1-Fc fusion proteins required for the pharmacokinetic and animal efficacy
*experiments described above are 250 μg EPO-IgG1-Fc and 500 μg G-CSF-IgG1-Fc. Using the COS cell transient
*expression system and Protein A affinity column, we purified ~250 μg EPO-IgG1-Fc and ~125 μg of G-CSF-
*IgG1-Fc from ~300 ml of conditioned medium collected by repeated harvests of seven T75 flasks. In order to
*obtain animal data for pharmacokinetics and efficacy of these molecules as soon as possible, we will scale up the
*COS cell transient expression system and Protein A affinity purification to produce sufficient material for the
*proposed *in vivo* experiments. Ultimately, we will ultimately want to express these fusion proteins in stably

*transformed mammalian cell lines such as CHO cells and these plans are described below. However, development
*of stable mammalian cell expression systems such as CHO cells typically employs multiple rounds of gene
*amplification to achieve high level expression of heterologous genes and this process can be time consuming.
*Therefore for the initial Phase II studies, we will produce the IgG1-Fc fusion proteins using the procedures
*described in the Phase I summary for transfections and harvesting of conditioned media.
*   For EPO-IgG1-Fc we will repeat the expression and purification of this protein from conditioned medium
*collected from 7 T75 culture flasks, which which should yield ~250 µg of protein. We still have in hand >100 µg
*of the EPO-IgG1-Fc protein that was purified during Phase I. Thus, one small (7 X T75) preparation should
*provide more than enough material for the initial Phase II *in vivo* experiments.
*   During Phase I we purified only 122 µg of G-CSF-IgG1-Fc from seven T75 culture flasks. For Phase II we plan
*to scale-up the preparation ~ 7-fold. We will use 24 T150 flasks for production of conditioned medium. Assuming
*results similar to those seen in Phase I, we project a recovery of ~850 µg which would be adequate. If needed, we
*will repeat the 24 X T150 scale preparation. Pooled material from two preps on this scale should provide sufficient
*material for the initial Phase II *in vivo* experiments.

*2. Non-Fused EPO and G-CSF Control Proteins
*   We will need to purify 250 µg rEPO and 450 µg rG-CSF for the pharmacokinetic and initial animal efficacy
*experiments described above. The small amount of EPO required will allow us to produce this protein by transient
*transfection of COS cells. G-CSF will be produced in transiently transfected COS cells or bacteria.
*   For expression of rEPO in transiently transfected COS cells. We will modify the 3' end of
*pCDNA3.1(+)::EPOfus (described above) to add DNA sequences encoding gly-gly-ser-asp-tyr-lys-asp-asp-asp-
*asp-lys followed by a translational stop codon. We will add this sequence as a synthetic ~40 bp double-stranded
*oligonucleotide with *Bam* HI – *Eco* RI ends to *Bam* HI – *Eco* RI cut pCDNA3.1(+)::EPOfus. This will generate
*an EPO construct with a carboxyterminal fusion of the 7 amino acid flexible linker present in the IgG fusions and
*the "FLAG" epitope. This construct will be expressed in COS cells. The FLAG epitope will allow use an affinity
*column based on the anti-FLAG monoclonal antibody M2 to purify this "FLAG-tagged" EPO. Using a
*baculovirus expression vector, we have expressed the same "FLAG-tagged" EPO protein in insect cells and
*purified it to homogeneity with an anti-FLAG monoclonal antibody M2 affinity column. This protein was fully
*active *in vitro* and we have greater than 200 µg of the purified protein in hand. We prefer to use this COS cell
*expressed EPO as a control for EPO-IgG1-Fc produced in COS cells to avoid differences in protein glycosylation
*that result from insect *versus* mammalian expression systems. However the baculovirus-produced material is
*available as backup if we encounter unexpected problems with COS cell expression of EPO. We will assess the
*level of EPO expression in transiently infected COS cells using an EPO ELISA and then scale the expression
*system to an appropriate level to generate sufficient material (~250 µg) for the Phase II *in vivo* experiments.
*   We have cloned and expressed human G-CSF in *E coli* as a protein secreted to the periplasm. We are currently
*purifying this molecule and have not as yet determined its *in vitro* bioactivity. If the protein is fully active, we
*should be able to use this system to purify sufficient *E. coli*-derived rG-CSF (~500 µg) for use in the *in vivo*
*studies. Naturally occurring G-CSF contains one O-linked glycosylation site and no N-linked glycosylation sites
*so that the distinction between *E coli* and COS derived proteins will be slight. Moreover the current G-CSF
*commercial product, Neupogen, is produced in *E coli*. If for unforeseen reasons we are unable to purify active G-
*CSF from *E coli*, we will modify the pCDNA3.1(+)::G-CSFfus plasmid to express a FLAG-tagged G-CSF via the
*same modifications described above for EPO. We do not know if a "FLAG-tagged" G-CSF will be fully active,
*but this seems likely since the carboxyterminal fusion of the much larger IgG1-Fc protein did not interfere with G-
*CSF bioactivity. If we use COS cell expression, we will assess the level of G-CSF expression in transiently
*transfected COS cells using a G-CSF ELISA and then scale the expression system to an appropriate level to
*generate sufficient material (~500 µg) for the Phase II *in vivo* experiments.

*II. Create Stably Transfected Mammalian Cell Lines Expressing the GF-IgG Fusion Proteins
*   Transient expression of the the GF-IgG fusion proteins using the COS cell expression system will provide the
*quickest route to *in vivo* testing of IgG fusion proteins for pharmacokinetics and animal efficacy. Ultimately,
*though, we expect to manufacture the proteins in stably transformed mammalian cell lines. Stably transfected
*CHO (Chinese Hamster Ovary) cell lines are widely used for recombinant protein expression (Geisse et al. 1996;
*Trill et al. 1995). High level expression of chromosomally integrated heterologous genes in CHO cells can be
*achieved by gene amplification. Typically the gene of interest is linked to a marker gene for which amplification is
*selectable. A number of genes that provide selections for amplification have been described (Kaufman 1990) but

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*murine dihydrofolate reductase (dhfr) is perhaps the most frequently employed. Amplification of this gene confers
*resistance to the folate analog methotrexate (MTX) and level of resistance is proportional to the dhfr gene copy
*number (Alt et al. 1978). Utility of this selection is enhanced by the availability of mutant CHO cell lines that are
*deficient in dhfr (Urlaub and Chasin, 1980). Typically a plasmid carrying the gene of interest and the murine dhfr
*gene is transfected into a dhfr⁻ CHO cell line and stable transformants selected using the dhfr⁺ phenotype or by
*resistance to a lethal drug such as G418 (geneticin sulfate), which is conferred by a plasmid-borne resistance gene.
*Neomycin phosphotransferase (NPT) is the most common gene used to confer resistance to G418. Subsequent
*multiple rounds of selection for resistance to increasing levels of MTX results in amplification of the plasmid-
*derived dhfr gene. In a fraction of the MTX resistant clones the level of expression of the gene of interest is
*correspondingly increased.
*   The procedures for transfection, selection and amplification in CHO cells are well described in the literature and
*have been used to express high levels of a number of immunoglobulin fusion molecules (Chamow and Ashkenazi
*1996) of a variety of heterologous proteins (Gelse, 1996; Kaufman, 1990). Amplification can typically yield cell
*lines producing 1-5 ug/ml of a desired recombinant protein in T flasks and 5 to 10-fold higher levels in roller bottle
*culture. Immunoglobulins such as monoclonal antibodies typically are expressed at relatively high levels in CHO
*cells (Trill et al 1995). Expression of our fusion protein in COS cells is on the order of ~ 1 ug / ml per 72 h, which
*is in the normal range for immunoglobulins such as monoclonal antibodies expressed in COS cells (Trill et al
*1995). Hopefully this will translate into robust expression our our fusion proteins in CHO cells as well, although
*COS cell results are not always predictive of results from expression in stable cell lines (Trill et al 1995).
*   We will construct expression vectors for EPO-IgG1-Fc and G-CSF-IgG1-Fc fusions. These vectors will incorporate the murine
*dhfr gene into the commercially available pCDNA3.1 expression vector (Invitrogen), which includes the NPT
*gene. The murine dhfr expression vector pdhfr2.9 is available from ATCC (# 37165). This plasmid expresses
*mouse dihydrofolate reductase in eukaryotic cell lines. The dhfr gene is selectable in dhfr⁻ CHO cell lines and can
*be amplified by standard selections for MTX resistance (Crouse et al, 1983). The dhfr coding sequence can be
*excised from pdhfr2.9 as a ~ 900 bp Bgl II fragment, which we will clone into the unique Bam HI site of the
*polylinker of the expression vector pREP4 (Invitrogen). This construct will position the dhfr coding sequence
*downstream of the strong RSV promoter, which is known to function in CHO cells (Trill et al, 1995) and upstream
*of a polyadenylation site deived from SV40. This dhfr expression cassette can then be conveniently excised from
*pREP4 as a Sal I fragment since Sal I sites closely flank the promoter and polyA addition site. Using
*olignucleotide linkers this Sal I fragment will be cloned into the unique Bgl II site of pCDNA3.1. The EPO-IgG1-
*Fc and G-CSF-IgG1-Fc genes will subsequently be cloned into the Hind III – Xba I. sites of pCDNA3.1 polylinker
*region under control of the CMV promoter.
*   Endotoxin free plasmid DNAs will be used to transfect dhfr⁻ CHO cells. We will obtain a dhfr⁻ CHO line, either
*CHO K1 DUKX B11 from L. Chasin at Columbia University or CHO duk⁻ from the ATCC (# CRL-9096). Cells
*will be cultured in F12/DMEM medim supplemented with 10% FCS, glutamine, glycine, hypoxanthine, and
*thymidine (Lucas et al., 199X). Transfections will be carried out with LipofectAMINE (Gibco BRL) using
*protocols similar to those used during Phase I. We will select for dhfr⁺ transfectants in F12/DMEM supplemented
*with 7% dialyzed FCS and lacking , glycine, hypoxanthine, and thymidine (Lucas et al., 199X). Alternatively, we
*will select for G418 resistance and subsequently screen transfectants for the dhfr⁺ phenotype. Dhfr⁺ clones will be
*expanded in selection medium and screened for EPO-IgG1-Fc or G-CSF-IgG1-Fc production by ELISA (R&D
*Systems). Clones with the highest expression levels will be pooled and subjected to multiple rounds of selection
*for MTX resistance at increaing drug concentration as described by Kaufman (1990). After each round of MTX
*selection, a set of individual clones will be tested for EPO-IgG1-Fc and G-CSF-IgG1-Fc production.

*III. Eliminate or minimize the linker in the EPO-IgG1-Fc and G-CSF-IgG1-Fc fusions.
*   We will determine the importance, in any, of the flexible peptide linker [ser-gly-gly-ser-gly-gly-ser] that fuses
*the growth factor domain to the immunoglobulin domain. We included the linker in our initial constructs to
*increase the likelihood that the fusions would be active. Typically, fusions to the IgG hinge region have not
*employed such flexible linkers, as the hinge itself functions in this capacity (Chamow and Ashkenazi, 1996). In
*principle there is no reason to include such a linker in a compound to be used in human subjects unless it is
*important for optimal functioning of the molecule. To determine if the peptide linker is important for biological
*activity of the EPO-IgG1-Fc and G-CSF-IgG1-Fc fusion proteins we will construct modified forms of these two
*fusions that shorten (to two or four residues) or eliminate the linker.
*   We will use PCR based "gene splicing by overlap extension" as described in Phase I to generate GF-IgG – Fc
*fusions without a linker. We will generate PCR products consisting of the IgG1-Fc coding sequence with a short
*5' extension, consisting of the 3' terminal ~ 15 bp of coding sequence of EPO or G-CSF fused directly to the hinge

*coding sequence. At the same time we will generate PCR products consisting of the EPO or G-CSF coding
*sequences with a short 3' extension consisting of the first 15 bp of the hinge coding sequence fused directly to the
*EPO or G-CSF coding sequence. The growth factor fragments and the IgG1-Fc fragments can then be spliced
*together via PCR "Sewing" to generate direct fusions. These PCR products will be digested with appropriate
*restriction enzymes to generate relatively small DNA segments that span the fusion point and which can be readily
*cloned into similarly cut vectors pCDN3.1(+)::EPO-IgG1-Fc and pCDNA3.1(+)::G-CSF-IgG1-Fc for sequence
*confirmation and COS cell expression. Cloning these smaller DNA fragments will minimize the sequencing that
*will need to be done to confirm the sequences of the direct fusions.
*   To construct a di-peptide [ser-gly] linker, we will PCR the IgG1-Fc sequence with a 5' oligonucleotide that adds
*the 5' extension CGCTCCGGA to the hinge coding sequence. The TCCGGA hexanucleotide is a cleavage site for
*the restriction endonuclease *Bsp* EI and encodes amino acids ser-gly. This PCR fragment will be digested with *Bsp*
*EI and *Sac* II and the ~ 240 bp fragment cloned into similarly cut pCDN3.1(+)::EPO-IgG1-Fc and
*pCDNA3.1(+)::G-CSF-IgG1-Fc. The unique *Bsp* EI site in each of these plasmids occurs at the first ser-gly in the
*linker [ser-gly-gly-ser-gly-gly-ser] so that the resulting recombinants will contain this 2 amino acid, ser-gly, linker.
*The sequence of the newly inserted ~ 250 bp *Bsp* EI – *Sac* II fragment will be verified.
*   A similar procedure will be used to construct the 4 amino acid [ser-gly-gly-ser] linker. We will PCR the IgG1-
*Fc sequence with a 5' oligonucleotide that adds the 5' extension CGCGGATCC to the hinge coding sequence. The
*GGATCC hexanucleotide is a cleavage site for the restriction endonuclease *Bam* HI and encodes amino acids gly-
*ser. This PCR fragment will be digested with *Bam* HI and *Sac* II and the ~ 240 bp fragment cloned into similarly
*cut pCDN3.1(+)::EPO-IgG1-Fc and pCDNA3.1(+)::G-CSF-IgG1-Fc. The unique *Bam* HI site in each of these
*plasmids occurs at the first gly-ser in the linker [ser-gly-**gly**-ser-gly-gly-ser] so the recombinants will contain the 4
*amino acid (ser-gly-gly-ser) linker. The sequence of the inserted ~ 250 bp *Bam* HI – *Sac* II peice will be verified.
*   During Phase I we measured specific activities of the IgG fusion proteins using purified proteins. In retrospect,
*we have found that combining bioactivity data from unpurified COS cell supernatants with quantitation of the IgG-
*Fc fusion proteins in the COS cell supernatants by ELISA gives specific activity results essentially identical to
*those obtained with the purified fusion proteins. This is the approach we will take to measure specific activities of
*the new linker constructs. The linker variants will be transfected into COS cells using the small-scale 6-well plate
*procedure described in the Phase I report. Concentrations of the IgG fusion proteins in the COS cell supernatants
*will be measured using EPO and G-CSF ELISAs. Bioactivities of the supernatants will be measured using the
*established bioassays described in the Phase I report. From these data we can determine specific activities of the
*new fusion constructs. The original linker constructs will be analyzed in parallel for controls. Those linker
*variants that appear to be fully active in this screen will be scaled up for purification and further characterization.

IV. Improve Bioactivities of IgG1-C$_H$ Fusion Proteins
*   The IgG-C$_H$ fusion constructs analyzed during Phase I appear to aggregate during purification and the specific
*activities of the fused growth factors were reduced ~ 2-3-fold as compared to the analogous Fc fusions.
*Aggregation may be due to hydrophobic interactions involving the CH1 domain that normally interfaces with the
*light chain. Coexpression of light chains may prevent aggregation. Most IgG fusions have employed the Fc
*portion of the immunoglobulin genes and it has been suggested that secretion and assembly of such fusions in the
*absence of light chain expression is improved by deletion of the CH1 domain (Chamow and Ashkenazi 1996). We
*are aware of only two IgG-C$_H$ fusions: CD4-IgG-C$_H$ (Capon et al, 1989) and IL-2- IgG-C$_H$ (Landolphi, 1994).
*Most IgG fusion molecules have been constructed as research tools to identify and characterize receptor – ligand
*interactions and the biological functions of these interacions (Chamow and Ashkenazi, 1996) and IgG-Fc fusions
*typically function well for the these types of experiments. Our focus with these fusions is on extending *in vivo* half
*life of the fused cytokine element and for this purpose complete heavy chain fusions, coexpressed with the light
*chain constant domain could potentially provide longer circulating half lives than the smaller Fc fusions. These
*larger, more complex structures will more closely resemble bona fide IgG molecules and as such may have
*circulating half lives closer to that of IgGs. All of the Fc fusion molecules for which there are *in vivo* data on
*circulating half life (Richert et al., 1999; Zeng et al., 1995) fall short of the 21 day half-life of the IgG molecule
*(Roitt et al., 1989). Therefore, during Phase II we will pursue active EPO and G-CSF fusions with IgG1-C$_H$ by
*cloning and coexpressing human light chains.
*   The DNA sequences of human kappa and lambda light chains are known (Heiter et al., 1980). We will clone
*the DNA sequences encoding the human kappa and / or lambda light chain constant (CL) regions by PCR
*amplification from the human leukocyte single-stranded cDNA (Clontech) used to clone the IgG1 and IgG4 heavy
*chain sequences during Phase I. We will verify the DNA sequences of the cloned CL domains and then use PCR
*based mutagenesis to modify the 5' and 3' ends for expression in mammalian cells in three different formats.

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*   **1.** We will add a Kozak sequence and a secretion signal to the 5' end to enhance translational initiation and
*direct the secretion of the light chain. We will add a translational stop codon to the 3' end of the sequence.
*Appropriate cloning sites will be added to the 5' and 3' ends to allow cloning into the mammalian cell expression
*vector pREP4 (Invitrogen) under control of the RSV promoter and preceding the SV40 derived polyA addition site.
*This construct will be used to cotransfect COS cells along with pCDNA3.1(+) derivatives that express EPO-IgG-
*C_H and G-CSF-IgG-$C_{H.}$ If we prefer to express both light and heavy chains from a single construct, we will excise
*the light chain sequence and the flanking promoter and polyA sites from pREP4 and clone this fragment into
*pCDNA3.1(+). The EPO-IgG-C_H and G-CSF-IgG-C_H coding sequences could then cloned into the pCDNA3.1(+)
*polylinker under control of the CMV promoter.
*   **2.** An alternative mode of light chain expression will be to modify the 5' end to add a portion of a flexible linker
*sequence fused to the amino-terminus of the CL coding sequence and add a translational stop codon to the 3' end
*of the sequence. Appropriate cloning sites will be added as well to the 5' and 3' ends to allow cloning as an in
*frame fusion to the EPO and G-CSF genes cloned in the plasmids pCDNA3.1(+)::EPOfus and pCDNA3.1(+)::G-
*CSFfus. We will cotransfect this plasmid into COS cells with plasmids that express EPO-IgG1-C_H and G-CSF-
*IgG1-$C_{H.}$ In this instance both heavy and light chains will contain growth factor fusions. The light and heavy
*chains could be expressed from a single pCDNA3.1 construct as described above.
*   **3.** A third mode of "co-expression" would be to modify the 5' and 3' ends of the CL coding sequence to
*incorporate portions of a flexible linker at both ends. By also incorporating appropriate cloning sites (*Bsp* EI and
**Bam* HI) such a construct can be inserted into the Bsp EI and *Bam* HI sites within the flexible linkers of the EPO-
*IgG-C_H and G-CSF-IgG-C_H fusions in pCDNA3.1(+). The resulting constructs would encode single polypeptide
*[EPO]-[CL]-[IgG-C_H] and [G-CSF]-[CL]-[IgG-C_H] fusions. The fusion of the carboxy-terminus of the light chain
*constant region to the amino-terminus of the heavy chain CH1 domain seems reasonable by analogy to single chain
*Fv polypeptides. Flexible peptide linkers of the (ser-gly-gly) motif on the order 14 to 20 residues in length have
*been used to fuse the carboxy-terminus of the light chain variable region to the amino-terminus of the heavy chain
*variable domain (Stewart et al., 1995).

EXHIBIT H

Project No. _____
106
Book No. _____ TITLE 4/30/99 Transfer pBST 180 Purification

6/8/99

From Page No.

Purify 8P0 Igb1 Fc from CM. This transfection was to scale
6 10 T150 flasks from 4/30/99

1. Thaw CM. Vol = 950ml
   Conc on YM30 membrane

			150 → ϕ to clear ppt then
St. Vol	950ml	after conc to 100 ml	pace on ice O/N
Conc	90ml	continued conc on 6/9/99.
Buffer Rxn c̄	5ml	
Final Vol	95ml	= 10X Conc

2. Load onto Equilib Prot A col 1ml Ht Trap.
   SR ~0.8ml/min
   Collect ST as 1 fraC

3. Wash c̄ 20mM NaPi pH 7.0   10 col vol
   SR 1ml/min   Collect as 1 frac

4. Elute c̄ 0.1M NaCitrate pH 3.0.   Collect 0.5ml frac into 0.1m
   SR 1ml/min   1M Tris pH 9.0

   √ pH of frac & Adj to ~7.0-7.5

Witnessed & Understood by me,	Date	Invented by	Date
May S R	2/2/99	David	6/16/99
		Recorded by	

To Page No. _____

Page No.____

BioLogic Run Report   Printed Date: 17-Jun-99

Method Name: protein A elution   Run Name: Protein A col 4/30/99 transfec pB Run Date: 11:44:15 AM 06-09-99
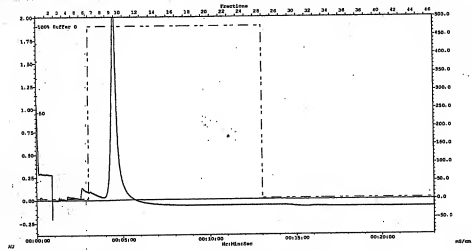
Sample: 4/30/99 transfection pBBT 180 EPO-IgG1-Fc
Column: Protein A 1ml HI trap
Buffer A: 20 mM NaPi, pH7.0
Buffer B: 0.1 M NaCitrate pH 3
Operator: Darin

Flow Rate: 1.0
Gradient:
Chart Speed:
Fraction Size: 0.5
Run Description:

1

After reviewing gels . Pool 9/10 9.10+11   Vol = 1.8 ml

Bradford Assay (p.109)   Conc is 382 µg/ml

Results

                                        Total protein ~687 µg

Final gel p110

Witnessed & Understood by me,   Date
Jay S. M   9/2/99

Invented by
David

Recorded by

Date
6/16/99

From Page No.___

6/9/9

B-16-70 SDS PAGE (Gel 1 N.R (Gel 2 RED)

Lane	Sample
1	BioRad Stnd
2	mt
3	Col load
4	4 T
5	J/S 7
6	8
7	9
8	10
9	11
10	12
11	13
12	14

Sample Prep
20 µl Sample + 5 µl SVSB 1-5% β2ME
heat 100°C 5 min   load All
Run on Gel at ~140 V C.V.

To Page No.___

Witnessed & Understood by me,   Date 9/2/99

Invented by   Date 6/16/99
Recorded by

EXHIBIT I

EPO Bioassay 7/6-9/99
Plate C

Protein (pg/ml)

- □— pBBT 185
- ○— pBBT 180

EPO Bioassay 7/6-9/99

Absorbance (490nm)

Protein (ng/ml)

- ◆— BV-EPO Stnd plt A
- ■— pBBT 185 plt A
- ●— pBBT 180 plt B

Plate C   7/9/99

pBBT 185

hi vue: 222.74, 24.7ng/ml = 1.084

lo vue: 0ng/ml = .052

$\frac{1.084 - .052 + .052}{2} = .568 = 1/2$

EC$_{50}$ = 2.1 ng/ml

pBBT 180

hi vue: 222.74ng/ml = 1.058

lo vue: 0ng/ul = .054

$\frac{1.058 - .054 + .054}{2} = .556 = 1/2$ tu

EC$_{50}$ = 1.3 ng/ml

EC$_{50}$'s

Sample	Plate	EC$_{50}$
BV-EPO	A	.48ng/ml
	B	.43 ng/ml
pBBT 185	A	2ng/ml
	C	2.1 ng/ml
pBBT 180	B	1.3 ng/ml
	C	1.3 ng/ml

To Page No._____

essed & Understood by me,

7 ay or

Date   7/12/99

Invented by   Danie

Recorded by

Date   7/15/99

EXHIBIT J

PROVISIONAL APPLICATION COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c)(2).

Docket Number	4152-3PROV	Type a plus sign (+) inside this box →	+

INVENTOR(s)/APPLICANT(s)

LAST NAME	FIRST NAME	MIDDLE INITIAL	RESIDENCE (CITY AND EITHER STATE OR FOREIGN COUNTRY)
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TITLE OF THE INVENTION (280 characters max)

"IMMUNOGLOBULIN FUSION PROTEINS"

CORRESPONDENCE ADDRESS

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STATE	Colorado	ZIP CODE	80203	COUNTRY	United States of America

ENCLOSED APPLICATION PARTS (check all that apply)

X	Specification	Number of Pages	66	X	Small Entity Statement
X	Drawing(s)	Number of Sheets	1		Other (specify)

METHOD OF PAYMENT (check all that apply)

X	A check or money order is enclosed to cover the filing fees		PROVISIONAL FILING FEE AMOUNT ($)	$75.00
X	The Commissioner is hereby authorized to charge filing fees and credit Deposit Account Number:	19-1970		

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☒ No.

☐ Yes, the name of the U.S. Government agency and the Government contract number are: _____
_____.

~~signature~~

Theresa A. Brown
Registration No. 32,547

Immunoglobulin Fusion Proteins

<u>Field of the Invention</u>

This invention relates generally to methods for constructing proteins and more specifically to methods for constructing recombinant IgG fusion proteins.

<u>Background of the Invention</u>

Prolonging the circulating half-lives of protein pharmaceuticals is of interest to patients and healthcare providers. Long acting protein therapeutics will require less frequent injections and should be effective at lower doses than proteins with shorter circulating half-lives. It is known that increasing the effective size of a protein can increase its circulating half-life by preventing removal of the protein by the kidney (Knauf et al., 1988; Mahmood, 1998). One method that can be used to increase the effective size of a protein is to use recombinant DNA technology to covalently fuse the protein of interest to a second protein. The larger fusion protein often has a longer circulating half-life than the non-fused protein (Capon et al., 1989; Zeng et al., 1995). One class of proteins that has been used frequently to create fusion proteins is immunoglobulins (Ig), which are major components of blood. Immunoglobulins occur in various classes known as IgG, IgM, IgA, IgD, and IgE (Roitt et al., 1989). Human IgGs can be further divided into various types known as IgG1, IgG2, IgG3 and IgG4, which are products of distinct genes. IgG1 is the most common immunoglobulin in serum (70% of total IgG) and has a serum half-life of 21 days (Capon et al., 1989; Roitt et al., 1989). Although less abundant, IgG4 also has a long circulating half-life of 21 days (Roitt et al., 1989)

Human IgGs have a multidomain structure, comprising two light chains disulfide-bonded to two heavy chains (reviewed in Roitt et al., 1989). Each light chain and each heavy chain contains a variable region joined to a constant region. The variable regions are located at the N-

terminal ends of the light and heavy chains. The heavy chain constant region is further divided into CH1, Hinge, CH2 and CH3 domains. The CH1, CH2 and CH3 domains are discreet domains that fold into a characteristic structure. The Hinge region is a region of considerable flexibility. Flexibility of the hinge can vary depending upon the IgG isotype (Oi et al., 1984; Dangl et al., 1989). IgG heavy chains normally form disulfide-linked dimers through cysteine residues located in the Hinge region. The various heavy chain domains are encoded by different exons in the IgG genes (Ellison et al., 1981; 1982).

Proteins have been fused to the heavy chain constant region of IgGs at the junction of the variable and constant regions (thus containing the CH1-Hinge-CH2-CH3 domains - referred to herein as the IgG-C_H fusions) at the junction of the CH1 and Hinge domains (thus containing the Hinge-CH2-CH3 domains - referred to herein as IgG-Fc fusions), and at the C-terminus of the IgG heavy chain (referred to herein as IgG-C-terminal fusions).

IgG fusion proteins have been created most often with the extracellular domains of cell surface receptors (reviewed in Chamow and Ashkenaki, 1996). Examples of extracellular domains of cell surface receptors that have been joined using recombinant DNA technology to the C_H or Fc domains of human or mouse IgGs include CD4 (Capon et al., 1989), tumor necrosis factor receptors (Mohler et al., 1993), CTLA4 (Linsley et al., 1991a), CD80 (Linsley et al., 1991b), and CD86 (Morton et al., 1996). Extracellular domains of receptors evolved to function when fused to other amino acids, i.e., the transmembrane and intracellular domains of the receptor; therefore it is not surprising that extracellular domains retain their ligand binding properties when fused to other protein domains such as IgG domains. Despite this, differences in ligand binding properties have been noted for certain extracellular domains. For example, a fusion protein comprised of the extracellular domain of CD4 to human IgG1-C_H had 2-fold reduced affinity for the CD4 ligand gp120 than non-fused CD4 (Capon et al., 1989).

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There are significantly fewer examples of proteins that are normally soluble, e.g., growth factors and cytokines, etc, which have been fused to IgG domains and retained full biological activity. Soluble proteins did not evolve to function when fused to other proteins and there is no reason to expect them to retain biological activity when fused to other proteins. In fact, in the majority of the published examples, biological activity of the fused cytokine/growth factor was significantly reduced relative to the non-fused cytokine/growth factor (see below). Whether or not the cytokine/growth factor will function properly when fused to another protein will depend upon many factors, including whether the amino-terminus or carboxy-terminus of the cytokine/growth factor is exposed on the surface of the protein, whether these regions are important for biological activity of the cytokine/growth factor and whether the cytokine/growth factor is able to fold properly when fused to another protein. By their very nature, such factors will be highly protein-specific. Results with the few growth factor/cytokine fusion proteins that have been studied have shown how protein-specific biological activity of the fusion protein can be. In the majority of cases, biological activity of the fused growth factor/cytokine is severely reduced, whereas, in the minority of cases full biological activity of the growth factor/cytokine is retained. In one case where biological activity of the fusion protein was significantly reduced, modifying the amino acids at the junction between the cytokine/growth factor and the IgG domain resulted in a fusion protein with improved biological activity. This same modification did not improve biological activity of a second cytokine fused to the same IgG domain (see below).

Growth factors that have been fused to IgGs include keratinocyte growth factor (KGF), fibroblast growth factor (FGF) and insulin-like growth factor (IGF-I). A KGF-mouse IgG1-Fc fusion protein was created by LaRochelle et al. (1995). On a molar basis, the fusion protein was 4-5-fold less active than KGF in stimulating proliferation of Balb/MK cells in an *in vitro*

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bioassay. The KGF-IgG-Fc fusion also had approximately 10-fold lower affinity for the KGF receptor on cells than did KGF. A fibroblast growth factor-human IgG-Fc fusion was constructed by Dikov et al. (1998). On a molar basis the FGF- IgG1-Fc fusion protein was approximately 3-fold less active than FGF in *in vitro* assays in stimulating DNA synthesis in NIH 3T3 cells. Shin and Morrison (1990) fused IGF-I to the C-terminus of IgG and found that the IGF-I-IgG C-terminal fusion protein had less than 1% of the *in vitro* biological activity of IGF-I.

Examples of cytokines that have been fused to IgG domains include IL-2, IL-4, IL-10 and GM-CSF. Landolphi (1991; 1994) described an IL-2-IgG1-C_H fusion protein, which included an extra amino acid, serine, between the C-terminus of IL-2 and the N-terminus of the IgG-C_H domain. The IL-2-IgG1-C_H fusion protein was as active, on a molar basis, in *in vitro* bioassays as IL-2, but no details were provided as to how protein concentrations were quantitated (Landolphi, 1991; 1994). Zeng et al. (1995) fused mouse IL-10 directly to the Fc region of mouse IgG2a; however the first amino acid of the Fc hinge region was changed from Glu to Asp. Zeng et al (1995) reported that the IL-10-IgG2a fusion protein was fully active in *in vitro* bioassays; however, only two concentrations of the fusion protein were studied, both of which were saturating. These high protein concentrations would have allowed only major differences (e.g., 100-fold) in bioactivities between the IL-10-mouse IgG2a fusion protein and IL-10 to be detected. To detect smaller differences in bioactivities, one needs to analyze serial dilutions of the proteins in *in vitro* bioassays and calculate EC_{50}s (the amount of protein required for half-maximal stimulation). EC_{50}s of the IL-10-IgG2a and IL-10 were not reported by Zheng et al. (1995). Chen et al.(1994) also constructed an IL-2-IgG fusion protein and reported that this fusion protein was fully active. Gillies et al. (1993) also reported creating a fully active IL-2 fusion protein comprising IL-2 fused to the C-terminus of an antiganglioside IgG antibody.

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Unexpectedly, Gillies et al. (1993) found that a fusion between the same antibody and GM-CSF displayed only 20% of wild type GM-CSF bioactivity. Chen et al. (1994) were able to create a fully active IgG-C-terminal- GM-CSF fusion protein by inserting four amino acids between the antibody molecule and GM-CSF. Unexpectedly, they reported that fusion of IL-4 to the same antibody using the same four amino acid linker resulted in an IL-4 protein with 25-fold reduced biological activity (Chen et al., 1994).

Qiu et al. (1998) described homodimeric erythropoietin (EPO) proteins in which two EPO proteins were fused together using flexible peptide linkers of 3-7 glycine residues. The peptide linker joined the C-terminus of one EPO protein to the N-terminus of the second EPO protein. In vitro bioactivities of the fusion proteins were significantly reduced (at least 4-10-fold) relative to wild type EPO (Qiu et al., 1998).

It appears that the amino acids at the junction between the growth factor/cytokine domain and the IgG domain can have a profound influence on the biological activity of the fused growth factor/cytokine.

In work described herein, the inventors disclose IgG-Fc fusion proteins of erythropoietin (EPO) and granulocyte colony-stimulating factor (G-CSF) that have biological activities, on a molar basis, comparable to non-fused (EPO) and G-CSF. In contrast, the inventors discovered that IgG1-C_H fusions of EPO and G-CSF had reduced specific activities (2-3-fold) relative to the non-fused proteins. The inventors also found that identical IgG-Fc and IgG1-C_H fusion constructs using growth hormone (GH) had significantly reduced (4 to 17-fold) biological activity relative to non-fused GH. These results provide further evidence as to the unpredictability of IgG fusion protein bioactivities.

IL-2, IL-4, GM-CSF, GH, EPO and G-CSF have nearly identical structures, comprising four alpha helices joined by loops (reviewed in Mott and Campbell, 1995). The characteristic

structure of these proteins is known as the four helix bundle. The four helix bundle structure is shared by a large number of other cytokines and growth factors, including GH, EPO, TPO, G-CSF, GM-CSF, M-CSF, IL-2, IL-3, IL-4, IL-5 IL-6, IL-7, IL-9, IL-11, IL-12, IL-13, IL-15, alpha, beta and gamma interferon, CNTF, LIF, and oncostatin M (reviewed in Mott and Campbell, 1995). The fact that these proteins share a conserved structure makes it all the more surprising that bioactivities of the proteins are affected so differently when they were fused to other proteins such as IgGs.

The idea of creating IgG fusion proteins with other cytokines and growth factors, and in particular EPO and G-CSF, are contemplated in patent applications EP 0 464 533 A and WO99/02709. The possibility of creating IgG fusion proteins with cytokines and growth factors also is disclosed by Landolphi (1994). None of these patents or patent applications present any data regarding expression, purification and bioactivities of the contemplated EPO-IgG and G-CSF-IgG fusion proteins. Landolphi (1994) presents bioactivity data for an IL-2-IgG1-C_H fusion protein, but not for any other IgG fusion protein. The EPO-IgG1-Fc fusion protein contemplated by EP 0 464 533 A would contain the two amino acid linker, ProGlu, between Asp165 of the EPO coding sequence and the beginning of the IgG1-Fc hinge region. The C-terminal amino acid in EPO, Arg166 would be deleted. EP 0 464 533 A does not provide any information as to how the G-CSF coding region would be joined to the IgG1-Fc hinge region. Bioactivity data for neither protein is provided in EP 0 464 533. WO 99/02709 discloses construction of an EPO-mouse IgG2a-Fc fusion protein, but not an EPO-human IgG fusion protein. Bioactivity data for the EPO-mouse IgG2a-Fc fusion protein are not presented. WO 99/02709 also does not provide details as to the source of the EPO cDNA or human IgG genes used to construct the contemplated fusion proteins or the precise amino acids used to join EPO to the IgG domain. The reference cited in WO 99/02709 (Steurer et al., 1995) also does not provide this information.

Accordingly, WO 99/02709 does not adequately teach how EPO should be joined to an IgG domain. WO 99/02709 postulates that a flexible peptide linker of 0-20 amino acids can be used to join EPO to the IgG domain. The amino acids to be used to create the flexible linkers are not specified by WO 99/02709. Qiu et al. (1998) reported that EPO fusion proteins joined by flexible linkers of 3-7 glycine residues have significantly reduced biological activities (4-10-fold) relative to wild type EPO. Thus it is clear from the literature that the precise amino acids used to construct the linker fusing the cytokine/growth factor to the second protein are critical for determining bioactivity of the fusion protein. Since a peptide linker of 20 amino acids can have 20^{20} possible sequences (since there are 20 different amino acid possibilities for each position in the sequence) it would require undo experimentation to test all possible linker sequence permutations to identify a linker that allows complete bioactivity of the cytokine/fusion protein to be retained.

In work described herein, the inventors discovered that bioactivities of fusion proteins also can vary depending upon the isotype of the IgG domain. This result was not predicted from the literature. The inventors postulate that the bioactivity variability thry observed may relate to the flexibility of the hinge region in the IgG domain used to construct the fusion proteins. Based upon these findings, the inventors believe that bioactivity of an EPO- mouse IgG fusion protein cannot be used to predict bioactivity of an EPO- human IgG fusion protein.

Brief Description of the Figure.

Figure 1 shows reduced and non-reduced SDS-PAGE analysis of purified GH-, G-CSF- and EPO-IgG-fusion proteins. Panel A shows purified Growth Hormone (GH)-IgG fusion proteins. Lane 1, molecular weight standards; Lanes 2 & 3 are bovine IgG standard at 1 & 2µg respectively, reduced; Lanes 4,5 & 6 are GH-IgG1-C_H, GH-IgG1-Fc, and GH-IgG4-Fc

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respectively, reduced; Lane 8, 2µg bovine IgG standard non-reduced; Lanes 9,10 & 11, are identical to lanes 4,5 & 6 except non-reduced. Panel B shows purified G-CSF-IgG fusion proteins. Lanes 1,2,3 & 8 are identical to those in Panel A; Lanes 4,5 & 6 are G-CSF-IgG1-C$_H$, G-CSF-IgG1-Fc and G-CSF-IgG4-Fc respectively, reduced; Lanes 9,10 & 11 are identical to lanes 4,5 & 6 except non-reduced. Panel C shows purified EPO-IgG fusion proteins. Lanes 1,2,3 & 8 are identical to those in Panel A; Lanes 4,5 & 6 are EPO-IgG1-C$_H$, EPO-IgG1-Fc and EPO-IgG4-Fc respectively, reduced; Lanes 9,10 & 11 are identical to lanes 4,5 & 6 except non-reduced.

<u>Detailed Description of the Invention</u>

The present invention provides methods for constructing novel EPO-IgG fusion proteins and G-CSF-IgG fusion proteins that possess *in vitro* biological activities equal to or within 2-fold, on a molar basis, the biological activities of non-fused EPO and G-CSF. The invention discloses other methods for constructing EPO and G-CSF-IgG fusion proteins that possess *in vitro* biological activities within 4-fold of non-fused EPO and G-CSF. The invention also discloses methods for constructing novel GH-IgG fusion proteins that possess specific *in vitro* bioactivities within 4-17-fold of non-fused GH. The invention further provides novel interferon and other cytokine IgG fusion proteins.

In the course of this work the inventors discovered that certain GH-, EPO- and G-CSF-IgG fusion proteins were secreted from mammalian cells as mixtures of monomers and disulfide-linked dimers. Previous studies had reported secretion of only disulfide-linked, dimeric IgG fusion proteins. For use as human therapeutics it will be desirable to use homogeneous populations of either monomeric or dimeric fusion proteins, but not mixtures of the two forms.

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The inventors disclose methods for purifying monomeric and dimeric forms of the fusion proteins.

The inventors also discovered that purified GH-, EPO- and G-CSF-IgG-C_H fusion proteins had reduced specific activities in *in vitro* biological assays compared to the non-fused proteins and IgG-Fc fusion proteins. This invention discloses methods to improve the specific biological activities of IgG-C_H fusion proteins.

The invention also discloses EPO-, G-CSF- and GH-IgG fusion proteins with certain specific activities in *in vitro* biological assays but which possess reduced ability to activate complement and bind Fc receptors.

The invention also discloses methods for constructing covalent, multimeric cytokine fusion proteins that retain biological activity.

The teachings of this application are meant to include variants of the disclosed proteins that possess some or all of the biological properties of the disclosed proteins. In some cases the variants may possess additional properties, e.g., improved stability or bioactivity, not shared by the original protein. For example, Mark et al. (1984) disclose an IFN-β mutein in which cysteine at position 17 is replaced by serine. IFN-β (Ser-17) displays improved stability relative to wild type IFN-β. Lu et al. (1992) disclose a G-CSF mutein in which cysteine ay position 17 is replaced by serine. Kuga et al. (1989), Hanazono et al. (1990) and Okabe et al. (1990) describe additional G-CSF muteins with enhanced biological properties. Elliot and Byrne (1995) have described mutants of EPO with enhanced biological activities.

The IgG fusion proteins described herein can be used to treat the same human diseases as the non-fused proteins. For example, the EPO-IgG fusion proteins can be used to treat anemia resulting from kidney failure, chemotherapy and drug complications. The EPO-IgG fusion

proteins also can be used to stimulate red blood cell formation in normal individuals who wish to enhance their blood volume prior to surgery. The G-CSF-IgG fusion proteins can be used to treat neutropenia resulting from chemotherapy and drug complications, for mobilization of progenitor cells for collection in peripheral blood progenitor cell transplants and for treatment of severe chronic neutropenia. The GH-IgG fusion proteins can be used to treat short stature and cachexia. The IFN-α-IgG fusion proteins can be used to treat viral diseases and cancer. The IFN-β-IgG fusion protein can be used to treat multiple sclerosis, viral diseases and cancer. The IgG fusion proteins described herein will possess longer circulating half-lives in patients, which will allow the fusion proteins to be administered less frequently or in effective lower doses than the non-fused proteins. Typically, GH and G-CSF are administered by daily injections and EPO is administered by thrice weekly injections. The IgG fusion proteins described herein also will be useful as diagnostic reagents for identifying cells expressing receptors for EPO, G-CSF and GH. The IgG fusion proteins also will be useful as *in vitro* reagents for studying cell proliferation and differentiation.

Example 1

Construction of Growth Factor-IgG Gene Fusions.

A. General Strategy.

Growth factor (GF)–IgG gene fusions were constructed as described below. The general strategy employed for these constructions is outlined here and the specifics of individual cloning steps are detailed below. Cloning of the IgG4-C_H coding sequence involved additional variations to the general strategy and these variations are described below. The human growth factor genes (GH, EPO and G-CSF) were cloned as cDNAs from various RNA sources detailed

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below. PCR primers used in these clonings added an optimized Kozak sequence (GCCACCC; Kozak, 1991) and a *Hin*d III restriction site to the 5' end of each these clones and a portion of a peptide linker (ser-gly-gly-ser) terminating in a *Bam* HI restriction site, to the 3' end of each of these clones. The growth factor genes were cloned as *Hin*d III – *Bam* HI fragments into the mammalian cell expression vector pCDNA3.1(+) (Invitrogen, Inc.) and sequenced. In parallel, IgG coding sequences (IgG1-Fc, IgG1-C$_H$, IgG4-Fc, IgG4-C$_H$) were cloned from cDNAs generated from human leukocyte RNA. PCR forward primers used in these clonings incorporated a portion of a peptide linker (gly-ser-gly-gly-ser) containing a *Bam* HI restriction site at the 5' end of each of these clones. The reverse PCR primers were designed to anneal to the 3' untranslated regions of the IgG1 and IgG4 mRNAs (about 40 bp downstream of the translational stop codon) and included an *Xba* I restriction site. The IgG coding sequences were cloned into pCDNA3.1(+) as *Bam* HI – *Xba* I fragments and confirmed by DNA sequencing. The fusion genes were then constructed by excising the IgG coding sequences as *Bam* HI – *Xba* I fragments and cloning these fragments into the pCDNA::GF recombinant plasmids that had been cut with *Bam* HI and *Xba* I. In the resulting pCDNA3.1 constructs the fusion genes are transcribed by the strong cytomeglovirus immediate early promoter present in pCDNA3.1(+) upstream of the cloned fusion gene. Ligation of the two fragments through the *Bam* HI site within the linker sequence results in a seven amino acid linker (ser-gly-gly-ser-gly-gly-ser) at the fusion junction.

pCDNA3.1	growth factor	linker	IgG	pCDNA3.1

↑ *Hin*d III   ↑ *Bam* HI   ↑ *Xba* I

B. Cloning of Growth Factor Genes

1. Cloning of human Growth Hormone : A cDNA encoding human Growth Hormone (GH) was amplified from human pituitary single-stranded cDNA (CLONTECH, Inc., Palo Alto, CA), using the polymerase chain reaction (PCR) technique and primers BB87 (5> GCAAGCTTGCCACCATGGCTACAGGCTCCCGGACG >3) and BB88 (5> CGCGGATCCTCCGGAGAA GCCACAGCTGCCCTCCAC >3). Primer BB87 anneals to the 5' end of the coding sequence for the hGH secretion signal, whereas the reverse primer, BB88, anneals to the 3' end of the GH coding sequence. The resulting ~ 680 bp PCR product was digested with *Hin*d III and *Bam* HI, gel purified and cloned into pCDNA3.1(+) vector (Invitrogen, Inc., Carlsbad, CA) that had been digested with *Hin*d III and *Bam* HI, alkaline phosphatase treated, and gel purified. A clone with the correct DNA sequence (Martial et al., 1979; Roskam and Rougeon, 1979; Seeburg, 1982; DeNoto et al., 1981) was designated pCDNA3.1(+)::GHfus or pBBT159.

2. Cloning of human Erythropoietin . A cDNA encoding human erythropoietin (EPO) was cloned by PCR using forward primer BB45 (5> CCCGGAT CCATGGGGGTGCACGAATGTCCTG >3) and reverse primer BB47 (5> CCCGAATTCTATGCCCAGGT GGACACACCTG >3). BB45 anneals to the DNA sequence encoding the initiator methionine and amino terminal portion of the EPO signal sequence and contains a *Bam* HI site for cloning purposes. BB47 anneals to the 3' untranslated region of the EPO mRNA immediately downstream of the translational stop signal and contains an *Eco* RI restriction site for cloning purposes. Total RNA isolated from the human liver cell line Hep3B was used in first strand synthesis of single-stranded cDNA for PCR. For preparation of total cellular RNA, Hep3B cells (available from the American Type Culture Collection, Rockville,

MD) were grown in Delbecco's Modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum (FBS). EPO expression was induced by treating the cells for 18 h with 130 µM Deferoxamine or 100 µM cobalt chloride. Both compounds have been shown to induce EPO mRNA and protein expression in Hep 3B cells (Wang and Semenza, 1993). RNA was isolated from the cells using an RNeasy Mini kit (Qiagen), following the manufacturer's directions. Approximately 320 µg of total RNA was isolated from 1.4×10^7 cells treated with cobalt chloride and 270 µg of total RNA isolated from 1.4×10^7 cells treated with Deferoxamine.

First strand synthesis of single-stranded cDNA was accomplished using a 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) from Boehringer Mannheim Corporation (Indianapolis, IN) and random hexamers were used as the primer. Subsequent PCR reactions using the products of the first strand syntheses as templates were carried out with primers BB45 and BB47. The expected ~ 600 bp PCR product was observed when reaction products were run out on an agarose gel. Both RNA preparations yielded an EPO PCR product. The PCR product was digested with *Bam* HI and *Eco* RI and cloned into vector pUC19 that had been cut with *Bam* HI and *Eco* RI and treated with alkaline phosphatase. DNA sequencing identified a clone containing the correct coding sequence for the EPO gene. This plasmid was designated pBBT131.

Plasmid pBBT131was used as template in a PCR reaction with primers BB89 (5>CGCAAGCTTGCCACCATGGGGGGTGC ACGAATGTCCT >3) and BB90 (5>CGCGGATCCTCCGGATCGTGTCCCCTGTCCTGCAGGC >3) to construct a modified EPO cDNA suitable for fusion with IgG genes. Primer BB89 anneals to the 5' end of the coding sequence for the EPO secretion signal and the reverse primer, BB90, anneals to the 3' end of the EPO coding sequence. The resulting ~ 610 bp PCR product was digested with *Hin*d III and *Bam*

HI, gel purified and cloned into pCDNA3.1(+) vector that had been digested with *Hin*d III and *Bam* HI, alkaline phosphatase treated, and gel purified. A clone with the correct DNA sequence (Lin et al., 1985) was designated pCDNA3.1(+)::EPOfus or pBBT176.

Similar procedures can be used to create modified EPO cDNAs in which Arg166 is deleted. In this case a primer with the sequence (5>CGCGGATCCTCCGGATCTGTCCCCTGTCCTG CAGGC >3) should be used in place of primer BB90.

3. Cloning of human Granulocyte Colony-Stimulating Factor. A cDNA encoding human Granulocyte Colony Stimulating Factor (G-CSF) was amplified by PCR from total RNA isolated from the human bladder carcinoma cell line 5637 (available from the American Type Culture Collection, Rockville, MD). The cells were grown in Roswell Park Memorial Institute (RPMI) 1640 media supplemented with 10% FBS, 50 units/ml penicillin and 50 μg/ml streptomycin. RNA was isolated from the cells using an RNeasy Mini RNA isolation kit purchased from Qiagen, Inc. (Santa Clarita, CA) following the manufacturer's directions. Approximately 560 μg of total RNA was isolated from 4.5×10^7 cells. First strand synthesis of single-stranded cDNA was accomplished using a 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) from Boehringer Mannheim Corp (Indianapolis, IN) and random hexamers were used as the primer. Subsequent PCR reactions using the products of the first strand synthesis as template were carried out with forward primer BB91 (5>CGCAAGCTTGCCACCATGGCTGGACC TGCCACCCAG>3 and reverse primer BB92 (5>CGCGGATCCTCCGGAGGGCTGGGCAAGGTGGCGTAG >3). Primer BB91 anneals to the 5' end of the coding sequence for the G-CSF secretion signal and the reverse primer, BB92, anneals to the 3' end of the G-CSF coding sequence. The resulting ~ 640

bp PCR product was digested with *Hin*d III and *Bam* HI, gel purified and cloned into pCDNA3.1(+) vector that had been digested with *Hin*d III and *Bam* HI, alkaline phosphatase treated, and gel purified. A clone with the correct DNA sequence (Souza et al., 1986; Nagata et al., 1986a,b) was designated pCDNA3.1(+)::G-CSFfus or pBBT165.
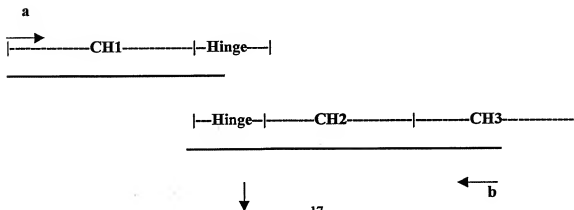
C. Cloning of IgG coding sequences.

1. Cloning of IgG1-Fc coding sequences.

A cDNA encoding IgG1-Fc (hinge-CH2-CH3 domains) was amplified from human leukocyte single-stranded cDNA (CLONTECH, Inc., Palo alto, CA) by PCR using primers BB83 (5>CGCGGATCCG GTGGCTCAGAGCCCAAATCTTGTGACAAAACT >3) and BB82 (5>CGCTCTAG AGGTACGTGCCAAGCA TCCTCG>3). Forward primer BB83 anneals to the 5' end of the coding sequence of the hinge domain of IgG1, whereas the reverse primer BB82 anneals to the 3' untranslated region of IgG1 and IgG4 mRNA ~ 45 bp downstream of the translational stop codon. The IgG1 and IgG4 sequences are identical over the 21 bp segment to which BB82 anneals. The ~ 790 bp PCR product was digested with *Bam* HI and *Xba* I, gel purified and cloned into pCDNA3.1(+) vector that had been digested with *Bam* HI and *Xba* I, alkaline phosphtase treated, and gel purified. Two clones were sequenced but each contained a single base pair substitution that resulted in an amino acid substitution mutation. Otherwise the sequences matched the published human IgG1 genomic DNA sequence (Ellison et al., 1982). The relative positions of the mutations in the two clones allowed us to use convenient unique restriction sites (*Sac* II in the CH2 domain of IgG1 and *Bgl* II in the pCDNA3.1(+) vector) to construct a full length IgG1-Fc clone in pCDNA3.1(+) via *in vitro* recombination. The resulting clone, which had the correct IgG1-Fc sequence, was designated pCDNA3.1(+)::fusIgG1-Fc or pBBT167.
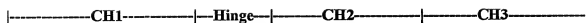
2. Cloning of IgG4-Fc coding sequences. A cDNA encoding IgG4-Fc (hinge-CH2-CH3 domains) was amplified from human leukocyte single-stranded cDNA (CLONTECH) by PCR using primers BB84 (5>CGCGGATCCGG TGGCTCGAGAGTCCAAATATGGTCCCCCATGC >3) and BB82 (5>CGCTCTAG AGGTACGTGCCAAGCA TCCTCG>3). Forward primer BB84 anneals to the 5' end of the coding sequence of the hinge domain of IgG4. The reverse primer BB82 is described above. The ~ 790 bp PCR product was digested with *Bam* HI and *Xba* I and cloned into pCDNA3.1(+) that had been similarly digested. A clone with the correct DNA sequence (Ellison et al., 1981) was designated pCDNA3.1(+)::fusIgG4-Fc or pBBT158.

3. Cloning of IgG1-C$_H$ coding sequences. A cDNA encoding IgG1-C$_H$ (CH1-hinge-CH2-CH3 domains) was amplified from human leukocyte single-stranded cDNA (CLONTECH) by PCR using BB81 (5>CGCGGATCC GGTGGCTCAGCCTCCACCAAGGGCCCATC>3) and BB82 (5>CGCTCTAGAGGTACGTGCCAAGC ATCCTCG>3). Forward primer BB81 anneals to the 5' end of the coding sequence of the CH1 domain of IgG1 and IgG4. The sequences at the 5' end of the CH1 domains of these two exons are almost identical: 19/20 nucleotides match. The reverse primer, BB82, is described above. The ~ 1080 bp PCR product was digested with *Bam* HI and *Xba* I, gel purified and cloned into pCDNA3.1(+) that had been digested similarly. These primers in principle could amplify both IgG1 and IgG4 sequences. Since IgG1 is much more abundant in serum than IgG4 (Roitt et al., 1989) we expected that most clones would encode IgG1. The first two clones sequenced were IgG1 but each contained a single base pair substitution that resulted in an amino acid substitution mutation. Otherwise the sequences obtained matched the published human IgG1 genomic DNA sequence (Ellison et al., 1982). The relative positions of the mutations in the two clones allowed us to use convenient unique

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restriction sites (*Age* I in the CH1 domain of IgG1 and *Bst* BI in the pCDNA3.1(+) vector) to construct a full length IgG1-C$_H$ clone in pCDNA3.1(+) via *in vitro* recombination. A clone with the correct IgG1-C$_H$ sequence was designated pCDNA3.1(+)::fusIgG1-C$_H$ or pBBT166.

4. Cloning of IgG4-C$_H$ coding sequences. The near identity of the DNA sequences encoding the 5' ends of the IgG1 and IgG4 CH1 domains and the relatively low abundance of the IgG4 mRNA led us to an alternative strategy for cloning the IgG4-C$_H$ coding sequences. We used PCR-based site directed mutagenesis to change the DNA sequence of the cloned IgG1 CH1 domain to match the amino acid sequence of the IgG4 CH1 domain. The CH1 domains differ at only 8 of 98 nucleotides and these positions are clustered, so that one round of PCR using two mutagenic oligos can convert the IgG1 CH1 sequence into the IgG4 CH1 sequence. A second round of PCR added the *Bam* HI site and linker sequence to the 5' end of the IgG4 CH1 and 21 bp of sequence from the IgG4 Hinge domain to the 3' end. The technique of "gene splicing by overlap extension" (Horton et al., 1993) was then employed to recombine the engineered IgG4 CH1 domain with the IgG4 Fc (Hinge-CH2-CH3) sequence. In this technique two separate fragments sharing a segment of identical sequence, the "overlap", at one end are extended through the annealed overlap regions in a PCR reaction as diagrammed below.

a

|————————CH1—————|–Hinge——|

|—Hinge—|———CH2————|———CH3————|

b

```
|-------------CH1-----------|---Hinge---|-------CH2-----------|--------CH3-------------|
```

To construct the IgG4 CH1 sequence, mutagenic primers BB119 (5> TCCACCAAG

GGCCCATCCGT CTTCCCCCTGGCGCCCTGCTCCAGGAGCACCTCCGAGAGC

ACAGC>3) and BB120 (5> TCTCTTG TCCACCTTGGTGTTGCTGGGCTTGTGATC

TACGTTGCAGGTGTAGGTCTTCGTCCCAA >3) were used in PCR reactions with

pBBT166, which carries the cloned IgG1-C$_H$ sequence as described above. Forward primer

BB119 anneals to the sequence encoding amino acids 2 through 23 of the CH1domain and

encodes 4 amino acid substitutions: S14C, K16R, G20E and G21S. Reverse primer BB120

anneals to the sequence encoding amino acids 76 through 97 of the CH1 domain and encodes 4

additional amino acid substitutions: Q79K, I82T, N86D and K97R. The ~ 290 bp product of this

PCR reaction was gel purified and used template in a PCR reaction with primers BB81(see

above) and BB121(5>TGGGGGACCATATTTG GACTCAACTCTCTTGTCCACCTT >3).

Reverse primer BB121 anneals to the 3' end of the CH1 domain of IgG4, adds amino acid 98 of

the CH1 domain and 21 bp extending into the Hinge domain of IgG4. The ~ 330 bp product of

this reaction was gel purified and used as one of the template molecules in the PCR splicing

reaction. The other template for the splicing reaction was generated by PCR of the cloned IgG4-

Fc sequence of pBBT158 (described above) with primers BB84 and BB82 which amplify the

IgG4 Fc domain as described above. The resulting ~ 790 bp product consists of the IgG4 hinge-

CH2-CH3 sequence. This fragment was gel purified and used as one of the template molecules

in the PCR splicing reaction. This reaction employed the primers BB81 and BB82 and generated

a full-length "spliced" product of ~ 1075 bp. To minimize the DNA sequencing required to

confirm this product, the PCR fragment was digested with *Bam* HI and *Sac* II and the ~ 530 bp fragment (containing the complete CH1 and hinge domains and a portion of the CH2 domain) was cloned into pBC-SK+ (Stratagene) for sequencing. The sequence of the *Bam* HI – *Sac* II fragment was confirmed for one clone which was the designated pBBT182. The *Bam* HI – *Sac* II fragment of pBBT182 was then used convert the GF–IgG4-Fc clones to full length GF–IgG4-C_H clones as detailed below.

D. Construction of Growth Factor–IgG (GF-IgG) fusions.

Most (9/12) of the growth factor-IgG gene fusions were generated by excising the IgG coding sequences cloned in pCDNA3.1(+) as *Bam* HI – *Xba* I fragments and cloning these fragments into the pCDNA::[GF] recombinant plasmids which had been cut with *Bam* HI and *Xba* I. The fusions of the three growth factor genes to IgG4-C_H were constructed by excising the ~ 530 bp *Bam* HI– *Sac* II fragment of pBBT182 and replacing the ~ 240 bp *Bam* HI- *Sac* II fragments of the three pCDNA::[GF]-IgG4-Fc clones. The resulting plasmids and the GF-IgG fusion proteins they encode are listed in Table 1.

Example 2

Expression and Purification of GF-IgG Fusion Proteins

A. **Small Scale Transfection of COS Cells**

Expression and bioactivity of the GF-IgG fusion proteins were assessed initially by small-scale transfection of COS cells. Endotoxin-free plasmid DNAs were prepared using an "Endo-Free Plasmid Purification Kit" (Qiagen, Inc.) according to the vendor protocol and used to transfect COS-1 cells (available from the American Type Culture Collection, Rockville, MD). The COS-1 cells were Delbecco's Modified Eagle's Media supplemented with 10% FBS,

50units/ml penicillin, 50μg/ml streptomycin and 2mM glutamine (growth media). Initial

transfection experiments were carried out in Costar 6 well tissue culture plates (VWR Scientific)

using the following protocol. Briefly, 2-3 x 10^5 cells were seeded into each well in 2 ml of

growth media and allowed to incubate overnight at 37°C and 5% CO_2 by which time the cells

had reached 50-60% confluency. For each well, 0.8 μg of plasmid DNA was complexed with 6

μl of LipofectAMINE reagent (Gibco BRL, Gaithersburg, MD) in 186 μl of OPTI-MEM I

Reduced Serum Medium (Gibco BRL, Gaithersburg, MD) for 30-45 minutes at room

temperature. COS-1 cells were washed one time with 2ml of OPTI-MEM I per well and then 1.8

ml of OPTI-MEM I was added to each well. The complex mixture was then added to the well

and left at 37°C, 5% CO_2 for approximately 4-5 hours. After the incubation period, the mixture

was replaced with 2 ml of growth media per well and left overnight at 37°C, 5% CO_2. The next

day the cells were washed twice with 2ml of DMEM (no additives) per well. Following the

wash steps, 2 ml of serum-free growth media was added to each well and the cells left at 37°C,

5% CO_2. Conditioned media containing the GF-IgG-fusion proteins were harvested after 72

hours and analyzed by SDS-PAGE and Western blot to confirm expression of the GF-IgG-fusion

proteins. The parent plasmid, pCDNA 3.1(+) (Invitrogen) was used as a negative control.

Transfection efficiency was estimated to be ~15%, using pCMVβ (Clontech), which expresses

E. coli β-galactosidase. Transfected cells expressing β-galactosidase were identified using a β-

Gal Staining Set (Boehringer Mannheim, Indianapolis, IN).

   Samples of the conditioned media were prepared in SDS-PAGE sample buffer with the

addition of 1% β-mercaptoethanol (BME) when desirable and electrophoresed on precast 14%

Tris-glycine polyacrylamide gels (Novex). Western blots using appropriate antisera

demonstrated expression of all of the GF-IgG fusion proteins (data not shown – see purified

proteins below). The GH-IgG fusion proteins were detected using a polyclonal rabbit anti-synthetic-hGH antiserum (kindly provided by Dr. A.F. Parlow and the National Hormone and Pituitary Program). The EPO- and G-CSF-IgG fusion proteins were detected using polyclonal antisera purchased from R&D Systems (Minneapolis, MN). Serial dilutions of the conditioned media were analyzed in the appropriate *in vitro* bioassays described later. These assays demonstrated significant activity in the conditioned media and encouraged us to perform large-scale transfections so that the proteins could be purified for specific activity measurements.

B. Large Scale Transfection of COS-1 Cells

Large scale transfections were carried out using Corning 100 mm tissue culture dishes or Corning T-75 tissue culture flasks (VWR Scientific). For 100 mm dishes, 1.6×10^6 cells were plated in 10 ml of growth media per dish and incubated at 37°C, 5% CO_2 overnight. For each 100 mm dish, 6.7 μg endotoxin-free plasmid DNA was complexed with 50 μl of LipofecAMINE reagent in 1.5 ml of OPTI-MEM I for 30-45minutes at room temperature. The COS-1 cells were washed one time with 10 ml OPTI-MEM per dish and then replaced with 6.6 ml of OPTI-MEM I. Following complex formation, 1.67 ml of the complex was added to each dish and left at 37°C, 5% CO_2 for 4-5 hours. After the incubation period, the reaction mixture was replaced with 10 ml of serum containing growth media per dish and left at 37°C, 5% CO_2 overnight. The next day the cells were washed twice with 10 ml of DMEM (no additives) per dish. Following the wash steps, 10ml of serum-free growth media was added to each dish and incubated at 37°C, 5% CO_2. Conditioned media were harvested routinely every three days (on days 3, 6, 9 and 12) and fresh serum-free growth media added to the cells. Transfections in T-75 culture flasks were identical to the 100mm dish protocol with the following exceptions: Cells were plated at 2×10^6

cells per flask and 9.35 µg of endotoxin-free plasmid DNA was complexed with 70 µl of

LipofectAMINE reagent in 2.1ml of OPTI-MEM I for each T-75 flask. Following complex

formation, 2.3 ml of the complex was added to each flask containing 7.7 ml of OPTI-MEM I.

Transfection efficiency was determined to be ~15% using pCMVβ and staining for β-

galactosidase expression as decribed earlier. The 12 plasmids listed in Table 1 were transfected

into COS-1 cells using the large-scale format to generate protein for purification. The

conditioned media were clarified by centrifugation and stored at -20°C for later purification.

Western blots were used to confirm expression of the IgG-fusion proteins.

C. Purification of GF-IgG-Fusion Proteins

Approximately 300 ml of transfected COS-1 cell conditioned media for each IgG-fusion

protein was pooled and concentrated using an Ultrafiltration cell and either a YM3 or YM30

DIAFLO Ultrafiltration membrane (Amicon, Beverly, MA). Concentrated pools were then

loaded onto a 1ml Pharmacia HiTrap recombinant Protein A column previously equilibrated with

20 mM NaPhosphate pH 7.0. The column was washed with 20 mM NaPhosphate until the A_{280}

had reached baseline. Bound protein was eluted with 100 mM NaCitrate pH 3.0 and collected

into sufficient 1M Tris pH 9.0 to achieve a final pH of approximately 7.0. Each fusion protein

was purified using a dedicated column to avoid any possibility of cross-contamination. All of

the IgG fusion proteins chromatographed similarly, yielding a single peak in the elution step.

Column fractions were analyzed using 8-16% precast Tris-glycine SDS-PAGE and fractions

enriched for the IgG-fusion protein were pooled. Protein concentrations of the pooled fractions

were determined by Bradford assay (Bio-Rad Laboratories, Richmond, CA) using bovine serum

albumin (BSA) as the standard. Recoveries of the various purified GF-IgG fusion proteins are given in Table 1 and ranged from 96 to 376 µg per 300 ml of conditioned media.

Figure 1 displays SDS gels of the purified proteins under reducing and non-reducing conditions and stained with Coomassie blue. All of the GF-IgG fusion proteins were recovered principally as disulfide-linked dimers. The molecular weights of the proteins ranged from 115-190 kDa kDa under non-reducing conditions and 50-70 kDa under reducing conditions, largely consistent with the molecular weights predicted in Table 1. The molecular weights of the EPO-IgG fusion proteins were the only ones larger than predicted (see Figure 1), presumably due to extensive glycosylation of the EPO domain. Monomeric fusion proteins were present in all of the samples, but were more abundant with the IgG4 fusion proteins (they can be seen in the non-reduced gels in Figure 1). The sizes of the major IgG fusion protein bands were different from the molecular weights of bovine IgG (see Figure 1), indicating that the proteins purified were not contaminating bovine IgGs from serum used in the experiments. The major IgG fusion protein bands also reacted with antisera specific for GH, EPO and G-CSF in Western blots of the samples. Purity of the IgG fusion proteins was estimated to be at least 90% from Coomassie blue staining of the gels.

All of the GF-IgG-CH fusions contained a large aggregate that migrated at the top of the gel when the samples were analyzed under non-reducing conditions. This aggregate disappeared when the samples were analyzed under reducing conditions and the amount of protein at the molecular weight of the major GF-IgG-C$_H$ bands seemed to increase proportionately. The aggregates also reacted with antisera specific for the various growth factors. These data suggest the aggregates are disulfide-linked multimers of the GF-IgG-C$_H$ fusion proteins. Under reducing SDS-PAGE conditions, all of the GF-IgG-C$_H$ fusions show a diffuse band approximately 20 kDa

larger than the main GF-IgG-C_H band. This band reacted with antisera against the growth factors and may be related to the aggregates.

Table 1   Predicted Molecular Weights and Recoveries of GF-IgG Fusion Proteins

Expression Plasmid	IgG-Fusion Protein	Predicted Molecular Weight (kDa) [1]		Protein Recovery
		Monomer	Dimer	μg / 300 ml
PBBT 171	GH-IgG1-C_H	58,706	117,412	376
PBBT 172	GH-IgG1-Fc	48,693	97,386	248
PBBT183	GH-IgG4-C_H	58,541	117,082	ND[2]
PBBT 163	GH-IgG4-Fc	48,365	96,730	96
PBBT 173	G-CSF-IgG1-C_H	55,564	111,128	122
PBBT 174	G-CSF-IgG1-Fc	45,551	91,102	122
PBBT 184	G-CSF-IgG4-C_H	55,399	110,798	ND
PBBT 175	G-CSF-IgG4-Fc	45,222	90,444	96
PBBT 179	EPO-IgG1-C_H	54,972	109,944	133
PBBT 180	EPO-IgG1-Fc	44,960	89,920	235
PBBT 185	EPO-IgG4-C_H	54,808	109,616	ND
PBBT 181	EPO-IgG4-Fc	44,632	89,264	257

[1] Does not include molecular weight contributions due to of glycosylation.

[2] Not determined

Example 3

In Vitro Bioactivities of Purified IgG Fusion Proteins

A. General Strategy

Cell proliferation assays were developed to measure *in vitro* bioactivities of the IgG fusion proteins. The assays measure uptake and bioreduction of the tetrazolium salt MTS [3-(4,5-dimethylthiazol-2-yl)-5-3-carboxyphenyl)-2-(4-sulphenyl)-2H-tetrazolium]. In the presence of

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an electron coupler such as phenazine methosulfate (PMS), MTS is converted to a formazan product that is soluble in tissue culture media and can be measured directly at 490 nm. Cell number is linear with absorbance values up to about 2. For EPO and G-CSF we were able to use existing cell lines to develop the bioassays. For GH, we needed to create a cell line that proliferates in response to GH. Such a cell line was created by stably transforming a murine leukemia cell line with a rabbit GH receptor.

In general, the bioassays were set up by washing the appropriate cells three times with media (no additives) and resuspending the cells at a concentration of $0.7-1 \times 10^5$/ml in media with additives (media used for each cell line is given below). Fifty µl ($3.5-5 \times 10^3$ cells) of the cell suspension was aliquotted per test well of a flat bottom 96 well tissue culture plate. Serial dilutions of the protein samples to be tested were prepared in serum-containing media. Fifty µl of the diluted protein samples were added to the test wells and the plates incubated at 37^0C in a humidified 5% CO_2 tissue culture incubator. Protein samples were assayed in triplicate wells. After 60-72 h, 20 µl of an MTS/PMS mixture (CellTiter 96 AQueous One Solution, Promega Corporation, Madison, WI) was added to each well and the plates incubate at 37^0C in the tissue culture incubator for 1-4 h. Absorbance of the wells was read at 490 nm using a microplate reader. Control wells contained media but no cells. Mean absorbance values for the triplicate control wells were subtracted from mean values obtained for the test wells. EC_{50}s, the amount of protein required for half maximal stimulation, was calculated for each sample and used to compare bioactivities of the proteins. Non-glycosylated molecular weights were used in the molar ratio calculations for consistency. Non-glycosylated molecular weights of 18,936, 18,987 and 22,129 were assumed for EPO, G-CSF and GH, respectively. Monomer molecular weights were used in the calculations for the IgG fusion proteins. Using molecular weights of the

monomer fusion proteins estimated from SDS gels (50-70 kDa) and glycosylated molecular weights of 35kDa for EPO and 19.7 kDa for G-CSF (GH is not glycosylated) gave similar activity ratios.

B. Bioactivities of EPO-IgG Fusion Proteins

The human UT7/epo cell line was obtained from Dr. F. Bunn of Harvard Medical School, Boston, MA. This cell line proliferates in response to EPO and is dependent upon EPO for cell survival (Boissel et al., 1993). The cells were maintained in Iscove's Modified Delbecco's Media (IMDM) supplemented with 10% FBS, 50 units/ml penicillin, 50 μg/ml streptomycin and 1 unit/ml recombinant human EPO (CHO cell-expressed; R&D Systems). Bioassays were performed in cell maintenance media using the procedures described above. Serial dilutions of recombinant CHO cell-expressed human rEPO (R&D Systems) were analyzed in parallel.

The UT7/epo cell line shows a strong proliferative response to rEPO, as evidenced by a dose-dependent increase in cell number and absorbance values. In the absence of rEPO, the majority of UT7/epo cells die, giving absorbance values less than 0.1. Commercial CHO cell-expressed rEPO had a mean EC_{50} of approximately 0.6 ng/ml in the bioassay (Table 2). This value agrees with EC_{50} values reported in the R&D Systems specifications (0.05 – 0.1 unit/ml or approximately 0.4-0.8 ng/ml). The EPO-IgG1-Fc and IgG4-Fc fusion proteins had identical EC50's of approximately 1.3 ng/ml in the bioassay (Table 2). On a molar basis, the EC_{50}s of CHO-cell expressed rEPO and the EPO-IgG-Fc fusions were identical (approximately 30 pM; Table 2). The EPO-IgG1-C_H fusion protein had an EC_{50} of 3.1 ng/ml or 60 pM (Table 2), which represents an approximate 2-fold reduction in specific activity relative to the EPO-IgG-Fc fusion proteins and non-fused rEPO. The EPO-IgG4-C_H fusion protein had a mean EC_{50} of 2.05 ng/ml.

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Table 2. Bioactivities of EPO-IgG Fusion Proteins

Clone	Protein	EC$_{50}$ Range (ng/ml) [1]	Mean EC$_{50}$ ng/ml	pM
-	RhEPO (CHO)	0.52, 0.55, 0.60	0.56	30
pBBT180	EPO-IgG1-Fc	1.1, 1.2, 1.5	1.27	28
pBBT181	EPO-IgG4-Fc	1.1, 1.2, 1.5	1.27	29
pBBT179	EPO-IgG1-C$_H$	2.9, 3.0, 3.5	3.13	57
pBBT185	EPO-IgG4-C$_H$	2.0, 2.1	2.05	37

[1] Data from individual experiments

C. Bioactivities of G-CSF-IgG Fusion Proteins

The murine NFS60 cell line was obtained from Dr. J. Ihle of the University of Tennessee Medical School, Memphis Tennessee. This cell line proliferates in response to human or mouse G-CSF or IL-3 (Weinstein et al., 1986). The cells were maintained in RPMI 1640 media supplemented with 10% FBS, 50 units/ml penicillin, 50 µg/ml streptomycin and 17-170 units/ml mouse IL-3 (R&D Systems). Assays were performed in cell maintenance media using the procedures described above. Serial dilutions of recombinant human G-CSF (*E. coli*-expressed; R&D Systems) were analyzed in parallel.

The NFS60 cell line shows a strong proliferative response to rhG-CSF, as evidenced by a dose-dependent increase in cell number and absorbance values. rhG-CSF had a mean EC$_{50}$ of 18 pg/ml in the bioassay (Table 3). This value agrees with the EC$_{50}$ value reported in the R&D Systems specifications (10-30 pg/ml). The G-CSF-IgG1-Fc and G-CSF-IgG4-Fc fusion proteins had mean EC$_{50}$'s of 34 and 50 pg/ml, respectively, in the bioassay (Table 3). On a molar basis, the EC$_{50}$s of rhG-CSF and the G-CSF-IgG1-Fc fusions were similar (approximately 0.9 pM; Table 3), whereas the EC$_{50}$ of the G-CSF-IgG4-Fc fusion protein was reduced slightly

(1.25 pM). The G-CSF-IgG1-C_H fusion protein had a mean EC_{50} of 182 pg/ml or 3.2 pM (Table 3), which represents an approximate 3-fold reduction in specific activity relative to G-CSF-IgG1-Fc fusion protein and non-fused rhG-CSF.

Table 3. Bioactivities of G-CSF-IgG Fusion Proteins

Clone	Protein	EC_{50} Range (pg/ml)[1]	Mean EC_{50} pg/ml	pM
-	rhG-CSF	17, 18, 18	17.7	0.93
PBBT174	G-CSF-IgG1-Fc	34, 39, 42	38.3	0.84
PBBT175	G-CSF-IgG4-Fc	50, 59, 61	56.7	1.25
PBBT173	G-CSF-IgG1-C_H	160, 190, 195	182	3.2
PBBT184	G-CSF-IgG4-C_H	ND [2]	-	-

[1] Data from individual experiments

[2] Not determined

D. Bioactivities of GH-IgG Fusion Proteins:

1. Development of an *in vitro* bioassay for hGH: We created a cell line that proliferates in response to GH by stably transforming a murine leukemis cell line, FDC-P1 with a plasmid that directs expression of a rabbit GH receptor. We cloned the rabbit GH receptor via RT-PCR from polyA+ rabbit liver RNA (CLONTECH) into pCDNA3.1(+). The resulting plasmid, termed pBBT118, was used to construct a stably tranformed FDC-P1 cell line that expresses the receptor. hGH binds human and rabbit GH receptors with similar affinities (Leung et al, 1987; Hammonds et al , 1991; Rowlinson et al., 1995). Rowlinson et al. (1995) described an hGH cell proliferation assay that uses the FDC-P1 cell line stably tranfected with the rabbit GH receptor.

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2. Cloning a cDNA Encoding the Rabbit GH Receptor: The rabbit GH receptor was cloned by
PCR using forward primer BB3 (5'-CCCCGGATCCGCCACCATGGATCTCTGG
CAGCTGCTGTT-3') and reverse primer BB36 (5'- CCCCGTCGACTCTAGAGCCATTA
GATACAAAGCTCT TGGG-3'). BB3 anneals to the DNA sequence encoding the initiator
methionine and amino terminal portion of the receptor. BB3 contains an optimized KOZAK
sequence preceding the initiator methionine and a *Bam* HI site for cloning purposes. BB36
anneals to the 3' untranslated region of the rabbit GH receptor mRNA and contains *Xba* I and
Sal I restriction sites for cloning purposes. Rabbit liver poly(A)$^+$ mRNA (purchased from
CLONTECH, Inc.) was used as the substrate in first strand synthesis of single-stranded cDNA to
produce template for PCR amplification. First strand synthesis of single-stranded cDNA was
accomplished using a 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) from Boehringer
Mannheim Corp. Parallel first strand cDNA syntheses were performed using random hexamers
or BB36 as the primer. Subsequent PCR reactions using the products of the first strand
syntheses as templates were carried out with primers BB3 and BB36. The expected ~ 1.9 kb
PCR product was observed in PCR reactions using random hexamer-primed or BB36-primed
cDNA as template. The random hexamer-primed cDNA was digested with *Bam* HI and *Xba* I,
which generates two fragments (~ 365 bp and ~ 1600 bp) because the rabbit GH receptor gene
contains an internal *Bam* HI site. Both fragments were gel-purified. The full-length rabbit GH
receptor cDNA was then cloned in two steps. First the ~1600 bp *Bam* HI - *Xba* I fragment was
cloned into pCDNA3.1(+) hat had been digested with these same two enzymes. These clones
were readily obtained at reasonable frequencies and showed no evidence of deletions as
determined by restriction digests and subsequent sequencing. To complete the rabbit receptor
cDNA clone, one of the sequenced plasmids containing the 1600 bp *Bam* HI - *Xba* I fragment
was digested with *Bam* HI, treated with Calf Alkaline Phosphatase, gel-purified and ligated with

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the gel purified ~365 bp *Bam* HI fragment that contains the 5' portion of the rabbit GH receptor gene. Transformants from this ligation were picked and analyzed by restriction digestion and PCR to confirm the presence of the ~365 bp fragment and to determine its orientation relative to the distal segment of the rabbit GH receptor gene. The sequence for one full length clone was then verified. This plasmid, designated pBBT118, was used to stably transfect FDC-P1 cells.

3. Selection of Stably Transfected FDC-P1 Cells Expressing the Rabbit GH Receptor:

Endotoxin-free pBBT118 DNA was prepared using a Qiagen "Endo-Free Plasmid Purification Kit" and used to tranfect FDC-P1 cells. The mouse FDC-P1 cell line was purchased from the American Type Culture Collection and routinely propagated in RPMI 1640 media supplemented with 10% fetal calf serum, 50 μg/ml penicillin, 50 μg/ml streptomycin, 2 mM glutamine and 17-170 Units/ml mouse IL-3 (FDC-P1 media). Mouse IL-3 was purchased from R&D Systems. FDC-P1 cells were transfected with plasmid pBBT118 using DMRIE-C cationized lipid reagent purchased from GIBCO, following the manufacturer's recommended directions. The next day transfected cells were transferred to T-75 tissue culture flasks containing 15 ml FDC-P1 media supplemented with IL-3 (17U/ml), hGH (5nM) and 10% horse serum rather than fetal calf serum. Horse serum was used because of reports that fetal calf serum contains a growth-promoting activity for FDC-P1 cells. Three days later the cells were centrifuged and resuspended in fresh FDC-P1 media containing 400 ug/ml G418, 17U/ml IL-3, 5nM hGH, 10% horse serum and incubated at 37^0C. Media was changed every few days. The cells from each transfection were split into T-75 tissue culture flasks containing fresh media and either mouse IL-3 (17 U/ml) or hGH (5nM). G418 resistant cells were obtained from both the IL-3- and hGH-containing flasks. The transformants used in the bioassays originated from flasks containing hGH. Twelve independent cell lines were selected by limiting dilution. Five of the cell lines (GH-R3, -R4, -

R5, -R6 and -R9) showed a good proliferative response to hGH. Preliminary experiments indicated that the EC_{50} for hGH was similar for each cell line, although the magnitude of the growth response varied depending upon the line. The GH-R4 cell line was studied in most detail and was used for the assays presented below. The cell lines were routinely propagated in RPMI 1640 media containing 10% horse serum, 50 units/ml penicillin, 50 μg/ml streptomycin, 2 mM glutamine, 400 μg/ml G418 and 2-5 nM pituitary hGH.

4. Bioassay results for Pituitary hGH and GH-IgG Fusion Proteins: Protein samples were assayed as described previously using RPMI media supplemented with 10% horse serum, 50 units/ml penicillin, 50 μg/ml streptomycin and 400 μg/ml G418. All assays included a human pituitary GH standard. Experiments utilizing the parental FDC-P1 cell line were performed in the same way except that the assay media did not contain G418 and FBS was substituted for horse serum. The parental FDC-P1 cell line shows a strong proliferative response to mouse IL-3, but not to pituitary hGH. In the absence of IL-3, the majority of FDC-P1 cells die, giving absorbance values less than 0.2. In contrast, FDC-P1 cells transformed with the rabbit growth hormone receptor proliferate in response to pituitary hGH, as evidenced by a dose-dependent increase in cell number and absorbance values. The EC_{50} for this effect ranged from 0.75-0.85 ng/ml pituitary hGH (0.03-0.04 nM) in different experiments, similar to what has been reported in the literature (Rowlinson et al., 1995). A significant difference between the parental FDC-P1 line and FDC-P1 cells transformed with the rabbit growth hormone receptor is that the latter cells survive in the absence of IL-3 or hGH, resulting in higher absorbance values (typically 0.6 – 1.1, depending upon the assay and length of incubation with MTS) in the zero growth factor control wells. The initial pool of rabbit growth hormone receptor transformants and all five independent

growth hormone receptor cell lines isolated showed the same effect. A similar result was obtained with a second set of independently isolated rabbit growth hormone receptor transfectants. Rowlinson et al. (1995) observed a similar effect, suggesting that IL-3/GH-independent survival is a consequence of the transformation procedure. Although the growth hormone receptor cell lines did not require IL-3 or hGH for growth, they still showed a robust proliferative response to IL-3 and hGH. The practical effect of the higher absorbance values in the absence of hGH is to decrease the "window" of the hGH response (the difference between the maximum and minimum absorbance values). This window consistently ranged from 30 - 100% of the zero growth factor values, similar to what was reported by Rowlinson et al. (1995).

All of the GH-IgG fusion proteins were active in the bioassay, but displayed reduced specific activities compared to pituitary hGH (Table 4). The GH-IgG1-Fc fusion protein was the most active, possessing an EC50 of 7-8 ng/ml (~0.16 nM). The GH-IgG4-Fc fusion protein was about two-fold less active than the IgG1 fusion protein, with a mean EC_{50} of 17 ng/ml (0.35 nM). The GH-IgG1-C_H fusion protein had the lowest activity, with an EC_{50} of 35 ng/ml (0.6nM). On a molar basis, bioactivities of the fusion proteins were reduced 4-fold (GH-IgG1-Fc), 10-fold (GH-IgG4-Fc) and 17-fold (GH-IgG1-C_H) relative to pituitary hGH.

Table 4. Bioactivities of GH-IgG Fusion Proteins

Clone	Protein	EC$_{50}$ Range (ng/ml) [1]	Mean EC$_{50}$ ng/ml	nM
-	Pituitary hGH	0.75, 0.75, 0.85, 0.85	0.8	0.036
PBBT172	GH-IgG1-Fc	6.5, 7.0, 7.8, 9	7.6	0.160
PBBT163	GH-IgG4-Fc	15, 16, 18, 18	17	0.350
PBBT171	GH-IgG1-C$_H$	28, 32, 40, 40	35	0.600
PBBT183	GH-IgG4-C$_H$	ND [2]	-	-

[1] Data from individual experiments

[2] Not determined

Example 4

Eliminate or Minimize the Linker in the GF-IgG Fusion Proteins.

GF-IgG fusion proteins in which the 7 amino acid linker [ser-gly-gly-ser-gly-gly-ser] that fuses the GF to the IgG domain is eliminated or reduced to 2 to 4 amino acids can be constructed as described below. Similar methods can be used to create linkers shorter than 7 amino acids, to create linkers longer than 7 amino acids and to create linkers containing other amino acid sequences. The experiments described below use IgG1-Fc and EPO and G-CSF as examples, however, similar procedures can be used for other GF or IgG domains, and domains for other IgG subtypes and domains from IgM, IgA,IgD and IgE antibodies. The modified fusion proteins can be expressed, purified and their specific activities determined in *in vitro* bioassays as described in **Examples 2 and 3.**

GF-IgG fusions without a linker can be created using PCR based "gene splicing by overlap extension" as described in **Example 1** (Horton et al., 1993). One can generate PCR products consisting of the IgG1-Fc coding sequence with a short 5' extension, consisting of the 3' terminal ~ 15 bp of coding sequence of EPO or G-CSF fused directly to the hinge coding

sequence. At the same time one can generate PCR products consisting of the EPO or G-CSF coding sequences with a short 3' extension consisting of the first 15 bp of the hinge coding sequence fused directly to the EPO or G-CSF coding sequence. The growth factor fragments and the IgG1-Fc fragments can then be spliced together via PCR "Sewing" (Horton et al., 1993) to generate direct fusions. These PCR products can be digested with appropriate restriction enzymes to generate relatively small DNA segments that span the fusion point and which can be readily cloned into similarly cut vectors pCDN3.1(+)::EPO-IgG1-Fc and pCDNA3.1(+)::G-CSF-IgG1-Fc for sequence confirmation and COS cell expression. Cloning these smaller DNA fragments will minimize the sequencing that will need to be done to confirm the sequences of the direct fusions.

To construct a di-peptide [ser-gly] linker, one can PCR the IgG1-Fc sequence with a 5' oligonucleotide that adds the 5' extension CGCTCCGGA to the hinge coding sequence. The TCCGGA hexanucleotide is a cleavage site for the restriction endonuclease *Bsp* EI and encodes amino acids ser-gly. This PCR fragment can be digested with *Bsp* EI and *Sac* II and the ~ 240 bp fragment cloned into similarly cut pCDN3.1(+)::EPO-IgG1-Fc and pCDNA3.1(+)::G-CSF-IgG1-Fc. The unique *Bsp* EI site in each of these plasmids occurs at the first ser-gly in the linker [ser-gly-gly-ser-gly-gly-ser] so that the resulting recombinants will contain this 2 amino acid, ser-gly, linker. The sequence of the newly inserted ~ 250 bp *Bsp* EI – *Sac* II fragment should be verified.

A similar procedure can be used to construct the 4 amino acid [ser-gly-gly-ser] linker. One can PCR the IgG1-Fc sequence with a 5' oligonucleotide that adds the 5' extension CGCGGATCC to the hinge coding sequence. The GGATCC hexanucleotide is a cleavage site for the restriction endonuclease *Bam* HI and encodes amino acids gly-ser. This PCR fragment can be digested with *Bam* HI and *Sac* II and the ~ 240 bp fragment cloned into similarly cut

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pCDN3.1(+)::EPO-IgG1-Fc and pCDNA3.1(+)::G-CSF-IgG1-Fc. The unique *Bam* HI site in each of these plasmids occurs at the first gly-ser in the linker [ser-gly-**gly-ser**-gly-gly-ser] so the recombinants will contain the 4 amino acid (ser-gly-gly-ser) linker. The sequence of the inserted ~ 250 bp *Bam* HI – *Sac* II piece should be verified.

Example5

Methods to Improve Bioactivities of IgG-C$_H$ Fusion Proteins

All of the IgG-C$_H$ fusion proteins appeared to aggregate during purification and the specific activities of the fused growth factors were reduced ~ 2-3-fold as compared to the analogous IgG-Fc fusions. Aggregation may be due to hydrophobic interactions involving the CH1 domain that normally interfaces with the light chain. Coexpression of IgG light chains with the GF-IgG-C$_H$ fusions should prevent aggregation. We describe three ways to coexpress IgG light and heavy chains below. The experiments described below use IgG-Fc, IgG-C$_H$, EPO and G-CSF as examples, however, similar procedures can be used for other GF or IgG domains, and domains for other IgG subtypes and domains from IgM, IgA,IgD and IgE antibodies. The DNA sequences of human kappa and lambda light chains are known (Heiter et al., 1980). cDNA sequences encoding the human kappa and / or lambda light chain constant (CL) regions can be obtained by PCR amplification from the human leukocyte single-stranded cDNA (Clontech) or human genomic DNA (Clontech). The DNA sequences of the cloned CL domains should be confirmed prior to use in the experiments described below. The modified fusion proteins can be expressed, purified and their specific activities determined in *in vitro* bioassays as described in Examples 2 and 3.

1. Co-expression of the light chain constant region. A Kozak sequence (Kozak, 1991) and a secretion signal can be added to the 5' end of the light chain constant region to enhance translational initiation and direct the secretion of the light chain constant region. A translational stop codon should be added to the 3' end of the sequence. Appropriate cloning sites can be added to the 5' and 3' ends to allow cloning into the mammalian cell expression vector pREP4 (Invitrogen) under control of the RSV promoter and preceding the SV40 derived polyA addition site. This construct can be used to cotransfect COS cells along with pCDNA3.1(+) derivatives that express, for example, EPO-IgG-C_H and G-CSF-IgG-C_H. Alternatively, both light and heavy chains can be expressed from a single plasmid construct. In this case, the light chain sequence and the flanking promoter and polyA sites from pREP4 should be excised with appropriate restriction enzymes and cloned into pCDNA3.1(+). The EPO-IgG-C_H and G-CSF-IgG-C_H coding sequences could then cloned into the pCDNA3.1(+) polylinker under control of the CMV promoter.

2. Co-expression of a GF-light chain constant region fusion proteins. An alternative mode of light chain expression would be to modify the 5' end to add a portion of a flexible peptide linker sequence fused to the amino-terminus of the CL coding sequence and add a translational stop codon to the 3' end of the sequence. Appropriate cloning sites can be added as well to the 5' and 3' ends to allow cloning as an in frame fusion to the EPO and G-CSF genes cloned in the plasmids pCDNA3.1(+)::EPOfus and pCDNA3.1(+)::G-CSFfus. This plasmid can be cotransfected into COS cells with plasmids that express, for example, EPO-IgG1-C_H and G-CSF-IgG1-C_H. In this instance both heavy and light chains will contain growth factor fusions. The light and heavy chains also could be expressed from a single pCDNA3.1 (+) construct as described above.

3. Light chain-heavy chain fusions. A third mode of "co-expression" would be to modify the 5' and 3' ends of the CL coding sequence to incorporate portions of a flexible peptide linker at both ends. By also incorporating appropriate cloning sites (*Bsp* EI and *Bam* HI) such a construct can be inserted into the Bsp EI and *Bam* HI sites within the flexible peptide linkers of the EPO-IgG-C$_H$ and G-CSF-IgG-C$_H$ fusions in pCDNA3.1(+). The resulting constructs would encode, for example, single polypeptide [EPO]-[CL]-[IgG-C$_H$] and [G-CSF]-[CL]-[IgG-C$_H$] fusions. The fusion of the carboxy-terminus of the light chain constant region to the amino-terminus of the heavy chain CH1 domain would be analogous to single chain Fv polypeptides. Flexible peptide linkers of the (ser-gly-gly) motif on the order 14 to 20 residues in length have been used to fuse the carboxy-terminus of the light chain variable region to the amino-terminus of the heavy chain variable domain (Stewart et al., 1995) and could be used to join the CL domain to the IgG-C$_H$ domain.

Example 6

GF-IgG Fusion Proteins with Reduced Complement Binding
and Fc Receptor Binding Properties

Certain GF-IgG1 fusion proteins may be toxic or lack efficacy in the animal models due to activation of complement or immune processes related to Fc receptor binding. For this reason, GF-IgG4 fusion proteins may be preferred because IgG4 is less efficient at complement activation and Fc receptor binding than is IgG1 (Roit et al., 1989). The EPO- and G-CSF-IgG4-Fc fusion proteins are as potent or nearly as potent as the IgG1-Fc fusion proteins in *in vitro* bioasays. Alternatively, one can perform *in vitro* mutagenesis experiments, as detailed below, to change specific amino acids in the IgG domains known to be responsible for complement

activation and Fc receptor binding. The modified fusion proteins can be expressed, purified and their specific activities determined in *in vitro* bioassays as described in Examples 2 and 3.

A. Complement Binding. Amino acids in IgGs that play a role in complement activation have been localized to the IgG CH2 domain. Specifically, amino acids Glu318, Lys320, Lys322, Ala330 and Pro331 in human IgG1 have been implicated as contributing to complement activation (Isaacs et al., 1998). Substitution of Glu318, Lys320 and Lys322 in IgG1 with alanine residues results in IgG proteins possessing reduced ability to activate complement (Isaacs et al., 1998). The amino acid sequence of IgG4 is identical to IgG1 in this region, yet IgG4 does not activate complement. As an alternative to using IgG4, one can change Glu318, Lys320 and Lys 322 (alone or in combination) of IgGs that have these residues to alanine residues or other amino acids that reduce complement activiation using the PCR-based mutagenesis strategies described in Example 1.

B. Fc Receptor Binding. Human IgG subclasses differ in their ability to bind Fc receptors and stimulate antibody-dependent cell-mediated cytotoxity (ADCC). IgG1, IgG3 and IgG4 are best at stimulating ADCC, whereas IgG2 has significantly reduced ability to stimulate ADCC (Roit et al., 1989). ADCC occurs through a mechanism that involves binding of the antibody to Fc receptors on immune cells. Amino acids responsible for Fc receptor have been localized to the CH2 domain of the IgG molecule. Specifically, amino acids 233-235 have been implicated in Fc receptor binding. Human IgG1 has the amino acid sequence GluLeuLeu in this region, whereas IgG2, which does not bind Fc receptors, has the sequence ProAlaVal. IgG4 has the sequence GluPheLeu in this region and is 10-fold less efficient at binding Fc receptors than IgG1. Substitution of the IgG2 sequence ProAlaVal for GluLeuLeu at positions 233-235 in IgG1or

IgG4 results in IgG1 and IgG4 antibodies with significantly reduced capacity for Fc receptor binding and ADCC (Isaacs et al., 1998). One can introduce these amino acid changes into the GF-IgG fusion protein constructs using the PCR-based mutagenesis strategy described in Example 1. Alternatively one can construct modified GF-IgG fusion proteins in which glycosylation of asparagine 297 in the IgG1 CH2 domain (or the equivalent asparagine residue in the other IgG subclasses) is prevented. Aglycosylated IgG1 antibodies display significantly reduced binding to Fc receptors and ability to lyse target cells as compared to glycosylated IgG1antibodies (Isaacs et al., 1998). One can construct aglycosylated versions of the GF-IgG fusion proteins by changing asparagine-297 to glutamine or another amino acid, or by changing threonine-299, which is part of the glycosylation recognition sequence (N-X-S/T), to alanine or to an amino acid other than serine. The amino acid in the X position of the glycosylation recognition sequence, i.e, amino acid 298, also could be changed to proline to prevent glycosylation of asparagine 297 in the IgG CH2 domain.

Example 7

Pharmacokinetic Experiments with GF-IgG Fusion Proteins

Pharmacokinetic experiments can be performed to demonstrate that the GF-IgG fusion proteins have longer circulating half-lives than the corresponding non-fused proteins. Both intravenous and subcutaneous pharmacokinetic data can be obtained. Terminal pharmacokinetic parameters can be calculated from the intravenous delivery data.

For the intravenous delivery studies, rats (~350g) should receive an intravenous bolus injection (0.1 mg/kg) of the IgG1-Fc fusion protein (EPO or G-CSF) or the corresponding non-fused protein (EPO or G-CSF) and circulating levels of the proteins measured over the course of 144 h. Three rats should be used for each protein sample. Blood samples should be drawn at 0,

0.08, 0.5, 1.5, 4, 8, 12, 24, 48, 72, 96, 120, and 144 h following intravenous administration. Serum levels of the test proteins can be quantitated using commercially available EPO and G-CSF ELISA kits (R & D Systems). Serial dilutions of each blood sample can be analyzed initially in the *in vitro* bioassays to identify dilutions that will fall within the linear range of the ELISAs. (0.025 to 1.6 ng/ml for EPO and 0.04 to 2.5 ng/ml for G-CSF). Titration experiments should be performed to determine the relative sensitivities of the ELISAs for detecting the IgG1-Fc fusion proteins and the corresponding non-fused proteins. This experiment will require 105 μg of each protein.

The subcutaneous delivery studies should follow the same protocol as the intravenous studies except for the route of delivery. Serum levels of the test proteins can be quantitated by ELISA as described above. This experiment will require 105 μg of each protein.

Example 8

Animal Efficacy Models

In vivo efficacy of the EPO-IgG1-Fc and G-CSF-IgG1-Fc fusion proteins can be demonstrated in normal rats and mice. These studies should use a variety of doses and dosing schedules to identify the proper doses and dosing schedules. Efficacy of the GF-IgG fusion proteins also can be demonstrated in appropriate disease models – anemia for EPO-IgG1-Fc and neutropenia for G-CSF-IgG1-Fc. The pharmacokinetic experiments will provide guidance in deciding dosing schedules for the IgG1 fusion proteins to be used for the animal studies. From published results with other IgG-Fc fusion proteins (Richter et al., 1999; Zeng et al., 1995) the GF-IgG fusion proteins should be effective when administered every other day or every third day and possibly less often, e.g. a single injection. The dosing schedules may have to be modified

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depending upon the results of the pharmacokinetic studies and initial animal efficacy results. The dose of protein administered per injection to the rodents also may have to be modified based upon the results of the pharmacokinetic experiments and initial animal efficacy results

A. EPO Animal Efficacy Models

In vivo efficacy of the EPO-IgG1-Fc fusion protein (and other EPO-IgG fusion proteins) can be determined in normal rodents. Wild type EPO is known to stimulate increases in hematocrit and erythropoiesis in normal rats or mice. The experiments described below uses rats. Sprague-Dawley rats (~200g) can be purchased from a commercial supplier such as Charles River (Wilmington, MA). Previous studies have shown that administration of 100 IU/kg (approximately 800 ng/kg) of rEPO once per day (160 ng SID / 200 g rat) by subcutaneous injection gives a significant increase in hematocrit and erythropoiesis in rodents (Matsumoto et al., 1990; Vaziri et al., 1994; Baldwin et al., 1998; Sykowski et al., 1998). Groups of 5 rats should receive subcutaneous injections of rEPO, EPO-IgG1-Fc or placebo (vehicle solution) at specified intervals for up to five days. A dose equivalent to a molar ratio of EPO (400 ng/200g rat of the EPO-IgG fusion protein) should be tested. Higher or lower doses also should be tested. A wide range of EPO-IgG1-Fc doses (over 500-fold variation) should be tested in these initial experiments to increase the likelihood that one of the doses will be effective. It is possible that administration of too much EPO-IgG1-Fc will impede erythropoiesis due to toxicity. Control rats should receive vehicle solution only. Additional control groups should receive rEPO (160 ng/SID for 5 days) and 160 ng rEPO using the same dosing regimen as EPO-IgG1-Fc. On day 6 the animals should be sacrificed and blood samples collected for hematocrit and complete blood cell count (CBC) analysis. Hematopoietic tissues (liver and spleen) should be collected, weighed and fixed in formalin for histopathologic analyses to look for evidence of increased

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erythropoiesis. Bone marrow should be removed from various long bones and the sternum for unit particle preps and histopathologic analysis to look for evidence of increased erythropoiesis. Comparisons between groups can be made using a Students T test for single comparisons and one-way analysis of variance for multiple comparisons. $P < 0.05$ should be considered significant.

Daily injections of rEPO should stimulate increases in hematocrit and erythropoiesis in the rats, whereas less frequent administration of the same dose of rEPO should not, or do so to a lesser extent. Dose-dependent increases in these parameters should be observed in the EPO-IgG-Fc-treated animals. Greater increases in these parameters may be observed in the EPO-IgG1-Fc-treated animals than in animals treated with EPO using the less frequent dosing schedules. Significantly less EPO-IgG1-Fc may be required to achieve the same increases in these parameters obtained with daily injections of EPO.

Additional experiments with less frequent dosing, e.g., a single injection, could be performed.

1. EPO Experiment 1 – Normal Rats – Every Other Day Dosing: Rats should receive injections every other day (EOD), i.e. on days 1, 3 and 5, for a total of three injections.

Group	Sample (Dose and Frequency)	Number of Rats	Protein Required
1	Vehicle solution (EOD)	5	0
2	EPO (160 ng SID)	5	4.0 µg
3	EPO (160 ng EOD)	5	2.4 µg
4	EPO-IgG1-Fc (0.64 ng EOD)	5	0.0096 µg
5	EPO-IgG1-Fc (3.2 ng EOD)	5	0.048 µg

6	EPO-IgG1-Fc (16 ng EOD)	5	0.24 µg
7	EPO-IgG1-Fc (80 ng EOD)	5	1.2 µg
8	EPO-IgG1-Fc (400 ng EOD)	5	6.0 µg

2. EPO Experiment 2 – Normal Rats – Every Third Day Dosing: Rats should receive injections every third day (ETD), i.e., on days 1 and 4, for a total of two injections.

Group	Sample (Dose and Frequency)	Number of Rats	Protein Required
1	Vehicle solution (ETD)	5	0
2	EPO (160 ng SID)	5	4.0 µg
3	EPO (160 ng ETD)	5	1.6 µg
4	EPO-IgG1-Fc (0.64 ng ETD)	5	0.0064µg
5	EPO-IgG1-Fc (3.2 ng ETD)	5	0.032 µg
6	EPO-IgG1-Fc (16 ng ETD)	5	0.16 µg
7	EPO-IgG1-Fc (80 ng ETD)	5	0.8 µg
8	EPO-IgG1-Fc (400 ng ETD)	5	4.0 µg

3. EPO Experiment 3 - Rat Anemia Model

Cisplatin-induced anemia is a well-characterized rodent model of chemotherapy-induced anemia and has direct relevance to the human clinical setting. rEPO reverses the anemia in this model when administered at daily doses of 100 Units/kg (Matsumoto et al., 1990; Vaziri et al., 1994; Baldwin et al., 1998). EPO-IgG-Fc should be effective at reversing anemia in this model. The dosing schedule for EPO-IgG-Fc to be used in this experiment culd be the one that worked best in the normal rat experiments. The experimental protocol outlined below assumes EPO-IgG-Fc is effective when administered ETD, but this can be altered based upon results of experiments with normal rats. Sprague-Dawley rats (~200g) should be treated on day 0 with an intraperitoneal injection of Cisplatin (3.5mg/kg) to induce anemia and randomized to various treatment groups. The dosing schedule and amounts of protein injected per rat should be as described for the ETD normal rat experiments described above. Rats should receive injections of EPO-IgG-Fc, rEPO or saline on days 1, 4 and 7, for a total of three injections. One control group of rats should receive daily subcutaneous injections of rEPO (100 Units/kg). Another control group should not receive the initial Cisplatin injection but should receive ETD injections of saline. On day 9 the rats should be sacrificed and blood and tissue samples obtained for comprehensive CBC and histopathology analyses.

Group	Cisplatin Treatment	Sample (Dose and Frequency)	Number of Rats	Protein Required
1	-	Vehicle solution (ETD)	5	-
2	+	Vehicle solution (ETD)	5	-
3	+	EPO (160 ng SID)	5	6.4 µg
4	+	EPO (160 ng ETD)	5	2.4 µg
5	+	EPO-IgG1-Fc (0.64 ng ETD)	5	0.0096µg
6	+	EPO-IgG1-Fc (3.2 ng ETD)	5	0.048 µg
7	+	EPO-IgG1-Fc (16 ng ETD)	5	0.24 µg
8	+	EPO-IgG1-Fc (80 ng ETD)	5	1.2 µg
9	+	EPO-IgG1-Fc (400 ng ETD)	5	6.0 µg

B. G-CSF Animal Efficacy Models

The G-CSF-IgG1-Fc experiments can be modeled after the EPO experiments described above. Mice or rats can be used for these experiments. The experiments described below uses mice. One can extrapolate pharmacokinetic data from the rat to the mouse because protein clearance is proportional to body weight (Mahmood, 1998). One can demonstrate efficacy of G-CSF-IgG1-Fc in normal animals using EOD or ETD dosing schedules. G-CSF has been shown to stimulate neutrophil levels in normal and neutropenic rodents at a dose of 100 µg/kg (Kubota et al., 1990; Kang et al., 1995), which is the standard dose that should be used for the experiments. Effectiveness of G-CSF-IgG1-Fc in a mouse neutropenia model can be

demonstrated using the optimum dosing schedule determined in the normal mouse experiments. Protein amounts required assume 20g mice are used.

Groups of 5 mice should receive subcutaneous injections of rG-CSF, G-CSF-IgG1-Fc or placebo (vehicle solution) at specified intervals for up to five days. One of the doses of G-CSF-IgG1-Fc should be a molar equivalent of rG-CSF. A wide range of G-CSF-IgG1-Fc doses (500--fold variation) should be used for these initial experiments to increase the likelihood that one of the doses will be effective. It is possible that administration of too much G-CSF-IgG1-Fc will impede granulopoiesis due to toxicity. Control rats should receive vehicle solution only. Additional control groups should receive rG-CSF (2 µg/SID for 5 days) and 2µg rG-CSF using the same dosing regimen as G-CSF-IgG1-Fc. On day 6 the animals should be sacrificed and blood samples collected for CBC analysis. Hematopoietic tissues (liver and spleen) should be collected, weighed and fixed in formalin for histopathologic analyses to look for evidence of increased granulopoiesis. Bone marrow should be removed from various long bones and the sternum for unit particle preps and histopathologic analysis to look for evidence of increased granulopoiesis. Comparisons between groups should be made using a Students T test for single comparisons and one-way analysis of variance for multiple comparisons. P< 0.05 should be considered significant.

Daily injections of rG-CSF should stimulate increases in circulating neutrophils and granulopoieis in the mice, whereas less frequent administration of the same dose of rG-CSF should not, or should do so to a lesser extent. Dose-dependent increases in these parameters should be observed in the G-CSF-IgG1-Fc-treated animals. Greater increases in these parameters may be observed in the G-CSF-IgG1-Fc-treated animals than in animals treated with rG-CSF using the less frequent dosing schedules. Significantly less G-CSF-IgG1-Fc may give the same increases in these parameters obtained with daily injections of rG-CSF.

1. G-CSF Experiment 1 – Normal Mice: Mice (~20g) should receive injections every other day (EOD), i.e. on days 1, 3 and 5, for a total of three injections. On day 6 the animals should be sacrificed and analyzed.

Group	Sample (Dose and Frequency)	Number of Mice	Potein Required
1	Vehicle solution (EOD)	5	0
2	G-CSF (2 µg SID)	5	50 µg
3	G-CSF (2 µg EOD)	5	30 µg
4	G-CSF-IgG1-Fc (0.008 µg EOD)	5	0.12 µg
5	G-CSF-IgG1-Fc (0.04 µg EOD)	5	0.6 µg
6	G-CSF-IgG1-Fc (0.2 µg EOD)	5	3 µg
7	G-CSF-IgG1-Fc (1 µg EOD)	5	15 µg
8	G-CSF-IgG1-Fc (5 µg EOD)	5	75 µg

2. G-CSF Experiment 2 – Normal Mice – Every Third Day Injection: Mice (~20g) should receive injections every third day (ETD), i.e., on days 1 and 4, for a total of two injections.

Group	Sample (Dose and Frequency)	Number of Mice	Protein Required
1	Vehicle solution (ETD)	5	0
2	G-CSF (2 µg SID)	5	50 µg
3	G-CSF (2 µg ETD)	5	20 µg
4	G-CSF-IgG1-Fc (0.008 µg ETD)	5	008 µg
5	G-CSF-IgG1-Fc (0.04 µg ETD)	5	0.4 µg
6	G-CSF-IgG1-Fc (0.2 µg ETD)	5	2 µg
7	G-CSF-IgG1-Fc (1 µg ETD)	5	10 µg
8	G-CSF-IgG1-Fc (5 µg ETD)	5	50 µg

Additional experiments with even less frequent dosing, e.g., a single injection, can be performed.

3. G-CSF Experiment 3 –Neutropenic Mice: Efficacy of G-CSF-IgG1-Fc also can be demonstrated in neutropenic animals.   Neutropenia can be induced by treatment with cyclophosphamide (CPA; 100 mg/kg), which is a commonly used chemotherapeutic agent that is myelosuppressive and relevant to the human clinical setting. G-CSF accelerates recovery of normal neutrophil levels in cyclophosphamide-treated animals (Kubota et al., 1990; Kang et al., 1995). Mice (~20g) should receive an intraperitoneal injection of cyclophosphamide on day 0 to

induce neutropenia. The animals should be divided into different groups, which will receive subcutaneous injections of G-CSF, G-CSF-IgG1-Fc or placebo. One control group should not receive cyclophosphamide but should receive placebo injections. The experiment described below assumes G-CSF-IgG1-Fc will be effective when administered ETD and that this is the dosing schedule that will be used for this experiment. The exact dosing schedule to be used will be determined by the results of the pharmacokinetic experiments and normal mouse efficacy studies described above. Mice should receive injections of test substances every third day (ETD), i.e., on days 1 and 4 for a total of two injections. On day six the animals should be sacrificed and blood and tissue samples analyzed as described above.

Group	CPA	Sample (Dose and Frequency)	Number of Mice	Protein Required
1	-	Vehicle solution (ETD)	5	0
2	+	Vehicle solution (ETD)	5	0
3	+	G-CSF (2µg SID)	5	50 µg
4	+	G-CSF (2 µg ETD)	5	20 µg
5	+	G-CSF-IgG1-Fc (0.008 µg ETD)	5	0.08 µg
6	+	G-CSF-IgG1-Fc (0.04 µg ETD)	5	0.4 µg
7	+	G-CSF-IgG1-Fc (0.2 µg ETD)	5	2 µg
8	+	G-CSF-IgG1-Fc (1 µg ETD)	5	10 µg
9	+	G-CSF-IgG1-Fc (5 µg ETD)	5	50 µg

Example 9

Separating IgG Fusion Protein Monomers from Dimers

The final purification scheme for the GF-IgG fusion proteins could include additional column chromatography steps in addition to affinity chromatography to remove protein contaminants. For use as human therapeutics it will be preferable to obtain preparations of GF-IgG monomers substantially free from GF-IgG dimers, and preparations of GF-IgG dimers substantially free from GF-IgG monomers. GF-IgG dimers can be separated from GF-IgG monomers using a variety of column chromatography procedures known to those with skill in the art. Examples of such procedures include ion-exchange, size exclusion, hydrophobic interaction, reversed phase, metal chelation, affinity columns, lectin affinity, hydroxy apatite and immobilized dye affinity chromatography. Other useful separation procedures known to those skilled in the art include salt precipitation, solvent precipitation/extraction and polyethylene glycol precipitation. Endotoxin levels in the purified proteins should be tested using commercially available kits to ensure that they are not pyrogenic.

Example 10

The procedures described in the preceding examples can, with minor modifications, be used to created IgG fusions with other proteins. Examples of other IgG fusion proteins that would find therapeutic uses in humans include IgG fusions of interferons alpha, beta and gamma, IL-11, TPO and GM-CSF. DNAs encoding these proteins can be cloned as described below and fused to the various IgG domains described in Example 1. The recombinant fusion proteins can be expressed and purified as described in Example 2. The purified fusion proteins can be tested in appropriate *in vitro* bioassays to determine their specific activities. DNA sequences, encoded amino acids and appropriate *in vitro* and *in vivo* bioassays for these proteins are well known in

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the art and are described in Aggarwal and Gutterman (1992; 1996), Aggarwal (1998), and Silvennoimem and Ihle (1996). Bioassays for these proteins also are provided in catalogues of commercial suppliers of these proteins such as R&D Systems, Inc, Endogen, Inc., and Gibco BRL.

1.Cloning human alpha interferon. Alpha interferon is produced by leukocytes and has antiviral, anti-tumor and immunomodulatory effects. There are at least 20 distinct alpha interferon genes that encode proteins that share 70% or greater amino acid identity. Amino acid sequences of the known alpha interferon species is given in Blatt et al., 1996). A "consensus" interferon that incorporates the most common amino acids into a single polypeptide chain has been described (Blatt et al, 1996). A hybrid alpha interferon protein may be produced by splicing different parts of alpha interferon proteins into a single protein (Horisberger and Di Marco, 1995). The following example describes construction of an alpha 2 interferon IgG fusion protein. Similar procedures can be used to create IgG fusions of other alpha interferon proteins.

DNA encoding human alpha interferon (IFN-α2) was amplified by PCR from human genomic DNA (CLONTECH). PCR reactions were carried out with BB93 (5>CGCGAATTCGGATATGTAAATAGATACACAGTG>3) and BB94 (5>CGCAAGCTTAAAAGATTTAAATCGTGTCATGGT>3) BB93 anneals to genomic sequences ~300 bp upstream (i.e. 5' to) of the IFN-alpha2 coding sequence and contains an *Eco* RI site for cloning purposes. BB94 anneals to genomic sequences ~100 bp downstream (i.e. 3' to) of the IFN-alpha2 coding sequence and contains a *Hin*d III site for cloning purposes. The PCR reaction employed 1X PCR reaction buffer (Promega Corp., Madison WI), 1.5 mM MgCl$_2$, 0.2 mM dNTPs, 0.2 μM of each oligonucleotide primer, 0.33 μg of genomic DNA and 0.4 units of Taq polymerase (Promega) in a 33μl reaction. The reaction consisted of 96°C for 3 minutes

followed by 35 cycles of: [95°C for 60 sec., 58°C for 75 sec., 72°C for 90 sec.] followed by

chilling the sample to 6°C. Reactions were carried out in a "Robocycler" thermal cycler

(Stratagene Inc. , San Diego, CA). The resulting ~ 1 kb PCR product was digested with *Eco* RI

and *Hin*d III and cloned into similarly digested, and alkaline phosphatased, pCDNA3.1(+)

(Invitrogen, San Diego, CA). A clone having the correct DNA sequence for IFN-α2 (Henco et

al, 1985) was identified and designated pBBT160.

   In order to construct and express gene fusions of IFN-α2 with IgG coding sequences the

IFN-α2 gene was modified at the 5' and 3' ends using PCR based mutagenesis. PBBT160

plasmid DNA was used as template for PCR with primers BB108 and BB109. PCR reactions

were carried out with forward primer BB108 (5'

CGCAAGCTTGCCACCATGGCCTTGACCTTT GCTTTA-3') and reverse primer BB109 (5'-

CGCGGATCCTCCGGATTCCTTACTT CTTAAACTTTC-3'). Primer BB108 anneals to the

5' end of the coding sequence for the IFN-α2 secretion signal and the reverse primer, BB109,

anneals to the 3' end of the IFN-α2 coding sequence. The resulting PCR product was digested

with *Hin*d III and *Bam* HI, gel purified and cloned into pCDNA3.1(+) vector that had been

digested with *Hin*d III and *Bam* HI, alkaline phosphatase treated, and gel purified. A clone with

the correct DNA sequence (Henco et al., 1985) was designated pCDNA3.1(+)::IFNAfus or

pBBT190 .

2. **Cloning human beta interferon.** Beta interferon is produced by fibroblasts and exhibits

antiviral, antitumor and immunoodulatory effects. Beta interferon is the product of a single

gene. DNA encoding human beta interferon (IFN-β) was amplified by PCR from human

genomic DNA (CLONTECH). PCR reactions were carried out with forward primer BB110 (5'-

CGCAAGCTTGCCACCATGACCAACAAGTGTCTCCTC-3') and reverse primer BB111 (5'-CGCGGATCCTCCGGAGTTTCGGAGGTAACCTGTAAG-3'). Primer BB110 anneals to the 5' end of the coding sequence for the IFN-β secretion signal and the reverse primer, BB111, anneals to the 3' end of the IFN-β coding sequence. The resulting PCR product was digested with *Hind* III and *Bam* HI, gel purified and cloned into pCDNA3.1(+) vector that had been digested with *Hind* III and *Bam* HI, alkaline phosphatase treated, and gel purified. A clone with the correct DNA sequence (Derynck et al., 1980) was designated pCDNA3.1(+)::IFNBfus or pBBT191.

3. Cloning human gamma interferon. Gamma interferon is produced by activated T cells and exhbits anti-viral, antitumor and immunomodulatory effects. A cDNA encoding human gamma interferon (IFN-γ) was amplified by PCR from total RNA isolated from the human Jurkat T cell line (available from the American Type Culture Collection, Rockville, MD). The cells were grown in RPMI media supplemented with 10% FBS, 50 units/ml penicillin and 50 μg/ml streptomycin. The cells were activated *in vitro* for 6 hours with 1 μg/ml PHA-L (Sigma chemical Company, catalogue L-4144) and 50 ng/ml PMA (phorbol 12-myristate 13-acetate, Sigma Chemical Company, catalogue # P-1585) to induce IFN-γ expression prior to RNA isolation (Weiss et al., 1984; Wiskocil et al., 1985). RNA was isolated from the cells using an RNeasy Mini RNA isolation kit purchased from Qiagen, Inc. (Santa Clarita, CA) following the manufacturer's directions. Approximately 104 μg of total RNA was isolated from 2.4×10^7 cells. First strand synthesis of single-stranded cDNA was accomplished using a 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) from Boehringer Mannheim Corp (Indianapolis, IN) and random hexamers were used as the primer. Subsequent PCR reactions using the products of

the first strand synthesis as template were carried out with forward primer BB112 (5'-
CGCAAGCTTGCCACCATGAAATATACAAGTTATATC-3') and reverse primer BB113 (5'-
CGCGGATCCTCCGGACTGGGATGCTCTTCGACCTTG-3'). Primer BB112 anneals to the

5' end of the coding sequence for the IFN-γ secretion signal and the reverse primer, BB113,

anneals to the 3' end of the IFN-γ coding sequence. The resulting PCR product was digested

with *Hin*d III and *Bam* HI, gel purified and cloned into pCDNA3.1(+) vector that had been

digested with *Hin*d III and *Bam* HI, alkaline phosphatase treated, and gel purified. A clone with

the correct DNA sequence (Gray et al., 1982) was designated pCDNA3.1(+)::IFNGfus or

pBBT192.

4. Construction of Interferon-IgG fusions. The IgG1-Fc coding sequence was fused to the

carboxyterminus of IFN-α2, IFN-β, and IFN-γ. The ~790 bp *Bam* HI – *Xba* I fragment was

excised from plasmid pBBT167 [described above in Example 1] and cloned into pBBT190,

pBBT191 and pBBT192 which had been digested with *Bam* HI and *Xba* I, and treated with

alkaline phosphatase. Similarly, IgG4-Fc coding sequence also was fused to IFN-α2, IFN-β, and

IFN-γ. The ~790 bp *Bam* HI – *Xba* I fragment of plasmid pBBT158 [described above in

Example 1] was excised, gel –purified and cloned into pBBT190, pBBT191 and pBBT192 which

had been digested with *Bam* HI and *Xba* I, and treated with alkaline phosphatase. The IgG1-C$_H$

coding sequence was fused to the carboxyterminus of IFN-α2, IFN-β, and IFN-γ. The ~1080 bp

Bam HI – *Xba* I fragment of plasmid pBBT166 [described above in Example 1] was excised and

cloned into pBBT190, pBBT191 and pBBT192 which had been digested with *Bam* HI and *Xba* I,

and treated with alkaline phosphatase. The structures of the resulting recombinant plasmids were

verified by restsriction endonuclease digestions and agarose gel electrophoresis. These plasmids and the IFN-IgG fusion proteins that they encode are listed in Table 5.

Table 5. Interferon – IgG Fusion Proteins

Expression Plasmid	IFN-Fusion Protein
PBBT193	IFN-α2-IgG1-Fc
PBBT194	IFN-α2-IgG4-Fc
PBBT220	IFN-α2-IgG1-C_H
PBBT195	IFN-β-IgG1-Fc
PBBT196	IFN-β-IgG4-Fc
PBBT221	IFN-β-IgG1-C_H
PBBT209	IFN-γ-IgG1-Fc
PBBT210	IFN-γ-IgG4-Fc
PBBT222	IFN-γ-IgG1-C_H

5. Bioactivities of Interferon-IgG Fusion Proteins.

In vitro biological assays for interferons include antiviral assays and cell proliferation inhibition assays. Proliferation of the human Daudi cell line (American Type Culture Collection, Rockville, MD) is inhibited by alpha, beta and gamma interferon and can be used to assay these proteins (Horoszewicz et al., 1979; Evinger and Pestka, 1981). Daudi cells are maintained in RPMI 1640 media supplemented with 10% FBS, 50 units/ml penicillin and 50 μg/ml streptomycin. Bioassays are performed in this media using the procedures described above except that the number of cells added to each well of a 96 well plate should be 5-20 x 10^3 and the

plates incubated at 37^0C for 3 to 4 days. The Daudi cells should be at early saturation density (1-2 x 10^6 cells/ml) before use in the assays for optimum effectiveness of the interferon. Serial dilutions of recombinant alpha, beta and gamma interferon (Endogen; R&D Systems; GibcoBRL, US Biological) should be analyzed in parallel. Recombinant alpha interferon has an IC_{50} (amount of protein to inhibit proliferation by 50%) of approximately 5-30 pg/ml.

Bioactivities of alpha, beta and gamma interferons also can be measured using viral plaque inhibition assays. These assays measure the ability of the interferon protein to protect cells from viral infection. Methods for performing these assays are described in Ozes et al.,(1992) and Lewis (1987; 1995). Human HeLa or WISH cells (available from the American Type Cuture Collection) should be plated in 96-well plates ($3x10^4$ cells/well) and grown to near confluency at 37^0C. The cells should be washed and treated for 24 hour with serial 2-3-fold dilutions of each IFN-IgG fusion protein preparation. Vesicular stomatitis virus (VSV) or encephalomyocarditis virus (EMCV) should then be added at a multiplicity of infection of 0.1 and the plates incubated for a further 24-48 hours at 37^0C. Additional controls should include samples without virus. When 90% or more of the cells have been killed in the virus-treated, no IFN control wells (determined by visual inspection of the wells), the cell monolayer should be stained with crystal violet (0.5% in 20% methanol) and absorbance of the wells read using a microplate reader. Alternatively, 20 µl of MTS/PMS mixture (CellTiter 96 AQueous One Solution, Promega Corporation, Madison, WI) can be added to the cell monolayers and absorbance the wells read at 490 nm after 1-4 hours later as described in Example 3. EC_{50} values (the amount of protein required to inhibit the cytopathic effect of the virus by 50%) should be used to compare the relative potencies of the fusion proteins and non-fused wild type proteins. Wild type IFN proteins protect cells from the cytopathic effects of VSV and EMCV and have

specific activities of approximately 1×10^7 to 2×10^8 units/mg in this assay, depending upon the IFN species (Ozes et al., 1992).

The IFN-α-IgG and IFN-β-IgG fusion proteins listed in Table 5 were expressed in COS cells and purified as described in Examples 1 and 2. Non-reducing SDS-PAGE analysis showed that the IFN-α-IgG1-Fc and IFN-α-IgG1-C_H fusion proteins consisted predominantly of disulfide-linked dimers; however small amounts of monomeric fusion protein was observed in all of the samples. The IFN-α-IgG1-C_H fusion protein also contained a significant amount of disulfide-linked aggregates, which failed to enter the gel. The IFN-α-IgG4-Fc fusion protein also was predominantly dimeric; more monomer was present in this sample than in the IFN-α-IgG1-Fc samples. The IFN-β-IgG1-Fc and IFN-β-IgG1-C_H fusion proteins also were largely dimeric, with small amounts of monomeric fusion protein present in each sample. In contrast, the majority of the purified IFN-β-IgG4-Fc fusion protein was monomeric; the remainder was dimeric. Significant amounts of disulfide-linked aggregates were present in all of the purified IFN-β-IgG fusion proteins.

The purified IFN-α-IgG and IFN-β-IgG fusion proteins were assayed using the Daudi cell growth inhibition assay described above. All of the IFN-α-IgG and IFN-β-IgG fusion proteins were biologically active. IC_{50} values for each protein were calculated and are shown in Table 6. Control recombinant IFN-α and IFN-β were purchased from Endogen, Inc. (Woburn, MA) and US Biological (Swampscott, MA), respectively.

Table 6. Bioactivities of IFN-α-IgG and IFN-β-IgG Fusion Proteins

Clone	Protein	IC$_{50}$ Range (ng/ml) [1]	Mean IC$_{50}$ (ng/ml)
-	rhIFN-α	0.015, 0.010	0.013
PBBT193	IFN-α2-IgG1-Fc	1.8, 2.5	2.1
PBBT194	IFN-α2-IgG4-Fc	2.5, 3.5	3.0
PBBT220	IFN-α2-IgG1-C$_H$	3.5	3.5
-	rhIFN-β	0.18, 0.3	0.24
PBBT195	IFN-β-IgG1-Fc	175, 200	188
PBBT196	IFN-β-IgG4-Fc	15, 15	15
PBBT221	IFN-β-IgG1-C$_H$	90	90

[1] Data from individual assays

6. Cloning IL-11. IL-11 stimulates evelopment of megakaryocyte precursors of platelets. A cDNA encoding human IL-11 can be amplified by PCR from RNA isolated from human cell lines that express IL-11 such as the human bladder carcinoma cell line 5367 and the HL60 and U937 leukemia cell lines (available from the American Type Culture Collection). PCR reactions can be carried out with forward primer IL-11F (5'-CGCAAGCTTGCCACCATGAACTGTGTTTGCCGCCTG –3') and reverse primer IL-11R (5'-CGCGGATCCTCCGGACAGCCGAGTCTTCAGCAGCAG –3'). Primer IL-11F anneals to the 5' end of the coding sequence for the IL-11 secretion signal and the reverse primer, IL-11R,

anneals to the 3' end of the IL-11 coding sequence. The resulting PCR product can be digested with *Hin*d III and *Bam* HI, gel purified and cloned into pCDNA3.1(+) vector that has been digested with *Hin*d III and *Bam* HI, alkaline phosphatase treated, and gel purified. Several clones should be sequenced to identify one with the correct DNA sequence. The human cell line TF-1 (available from the American Type Culture Collection) can be used to measure bioactivities of the IL-11-IgG fusion proteins.

7. Cloning TPO. Thrombopoietin (TPO) stimulates development of megakaryocyte precursors of platelets. A cDNA encoding human TPO can be amplified by PCR from single-stranded cDNA prepared from liver or kidney, which is available from commercial sources such as CLONTECH and Stratagene, Inc.. PCR reactions can be carried out with forward primer TPOF (5'- CGCAAGCTTGCCACCATGGAGCTGACTGAATTGCTC –3') and reverse primer TPOR (5'- CGCGGATCCTCCGGACCCTTCCTGAGACAGATTCTG –3'). Primer TPOF anneals to the 5' end of the coding sequence for the TPO secretion signal and the reverse primer, TPOR, anneals to the 3' end of the TPO coding sequence. The resulting PCR product can be digested with *Hin*d III and *Bam* HI, gel purified and cloned into pCDNA3.1(+) vector that has been digested with *Hin*d III and *Bam* HI, alkaline phosphatase treated, and gel purified. Several clones should be sequenced to identify one with the correct DNA sequence. The cell line MO7e (Avanzi et al., 1988) can be used to measure TPO bioactivity.

8. Cloning GM-CSF: A cDNA encoding human GM-CSF can be amplified by PCR from RNA isolated from a cell line that expresses GM-CSF such as the human T cell line HUT 102 (available from American Type Culture Collection) or from human peripheral blood lymphocytes the human Jurkat T cell line that had been activated with 20 µg/ml concanavalin

A (Sigma Chemical Company) and 40 ng/ml phorbol myristate acetate (PMA, Sigma Chemical

Company). PCR reactions can be carried out with forward primer GMCSFF (5'-

CGCAAGCTTGCCACCATGTGGCTGCAGAGCCTGCTG-3') and reverse primer GMCSFR

(5'- CGCGGATCCTCCGGACTCCTGGACTGGCTCCCAGCA 3'). Primer GMCSFF

anneals to the 5' end of the coding sequence for the GM-CSF secretion signal and the reverse

primer, GMCSFR, anneals to the 3' end of the GM-CSF coding sequence. The resulting PCR

product can be digested with *Hin*d III and *Bam* HI, gel purified and cloned into pCDNA3.1(+)

vector that has been digested with *Hin*d III and *Bam* HI, alkaline phosphatase treated, and gel

purified. Several clones should be sequenced to identify one with the correct DNA sequence .

Bioactivity of the GM-CSF-IgG fusion proteins can be measured using the TF-1 cell line

(available from the American Type Culture Collection).

Example 11
Multimeric Cytokine and Growth Factor fusion Proteins

Bioactive fusion proteins also can be created by constructing multimeric fusion proteins

of the growth factors and cytokines mentioned in this application. These multimeric fusion

proteins can be constructed as described for the IgG fusion proteins except that a second growth

factor/cytokine protein can be substituted for the IgG domain. The two growth factors/cytokines

can be joined together with or without linker amino acids between the two growth

factors/cytokines. Suitable peptide linkers include those described in Examples 1 and 4. The

fusion proteins can be homodimeric, heterodimeric, homomultimeric (comprising three or more

copies of the same growth factor/cytokine) or heteromultimeric (comprising two or more

different growth factors/cytokines). The most carboxy-terminal cytokine/growth factor domain

should be modified using procedures such as PCR to delete the protein's natural signal sequence

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and add, if desired, a short peptide linker sequence preceding the first amino acid of the mature protein sequence. The linker sequence could include a restriction enzyme site to facilitate joining to the amino-terminal cytokine/growth factor domain. In multimeric fusion proteins the cytokine/growth factor domains not at the amino- or carboxy-terminus of the protein can be modified to delete the natural signal sequence and termination codon and add, if desired, peptide linkers to the amino- and carboxy-termini of the protein. The fusion proteins can expressed in COS cells following transfection, purified and tested in appropriate *in vitro* bioassays.

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Figure 1.